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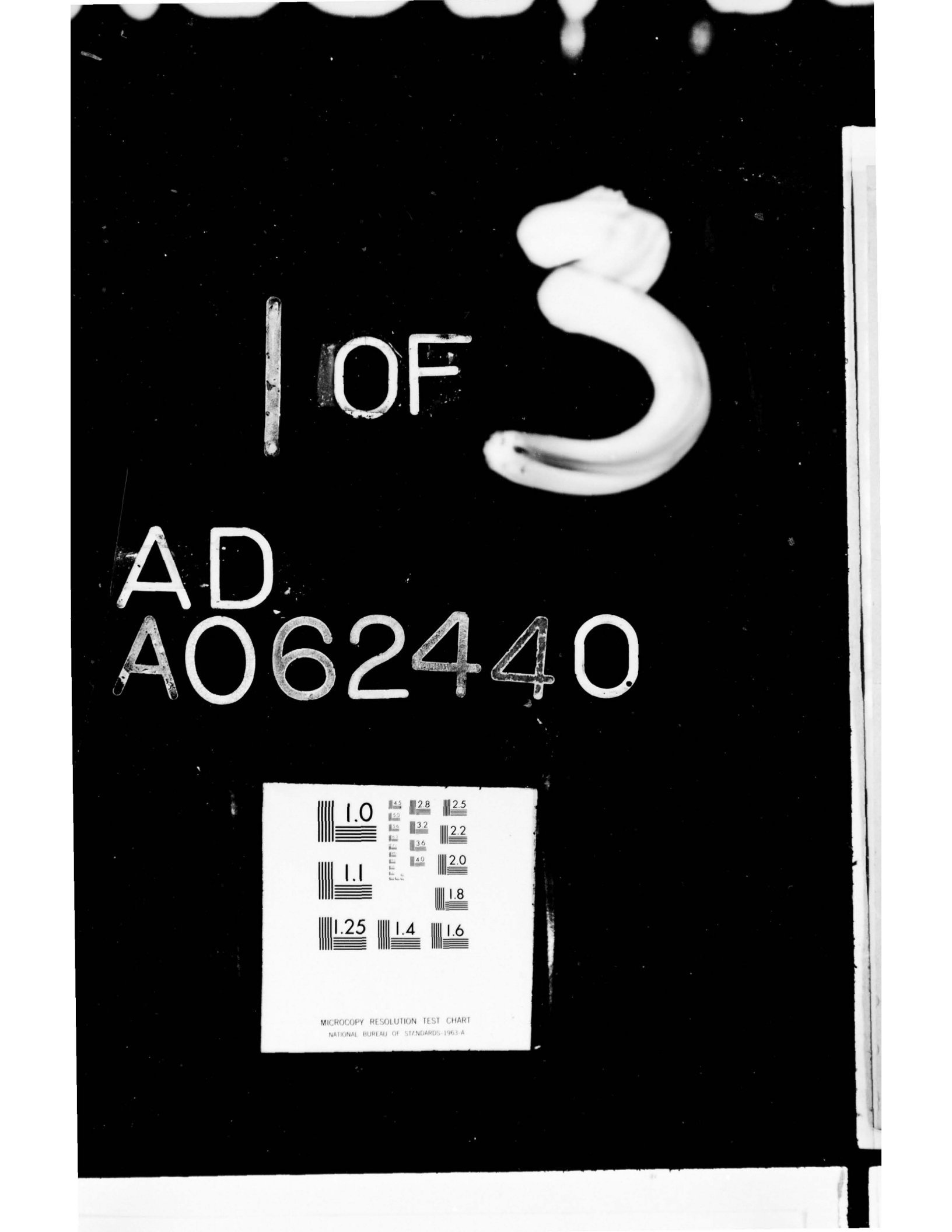
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# INVESTIGATION OF HEMATOLOGIC AND PATHOLOGIC RESPONSE TO DECOMPRESSION

Final Technical Report for Office of Naval Research
Contract N00014-71-C-0273 funded by the
Naval Medical Research and Development Command

INVESTIGATORS

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Submitted to:

Office of Naval Research
Department of the Navy



by

Virginia Mason Research Center
Seattle, Washington

May 10, 1978

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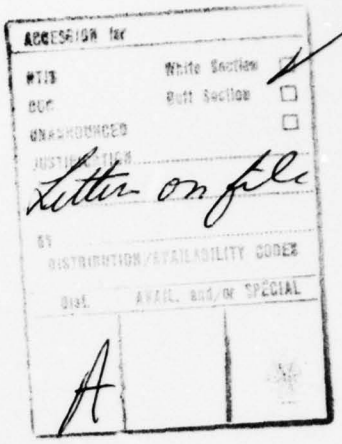


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General Statement and Summary

Knowledge of the pathology associated with inadequate decompression is truly embryonic. In that the Bureau of Medicine and Surgery, U.S.N., and Office of Naval Research saw fit to support our investigative effort, significant information was gathered which describes certain pathological mechanisms and hints at the description of etiological processes.

Our investigations evaluated the response of 67 miniature swine to 4760 hours of simulated diving in our chambers. The study included the testing of 1404 blood samples for kinetic measurements of hemostatic mechanisms, 425 radiographs for detection of bone disease and numerous histologic slides for microscopic examination.

Some of the information generated revealed the importance of endothelial integrity, its susceptibility to injury following inadequate decompression and its effect on the hemostatic mechanism and surrounding tissues when they are damaged. Two simultaneous but separate studies demonstrated the importance of collagen in the pathogenic process of osteonecresis.

Of significance is that this pathologic process was not limited to bone tissue, but probably occured throughout the body. Only the manifestation seems to differ in both degree and character.

Certain evidence accumulated appeared important and unequivocal:

(1) Dysbaric osteonecrosis was produced in miniature swine following a single decompression exposure.

(2) Hemostatic changes seen in diving animals and humans were the result of the decompression process, and more specifically, the relative tissue inert gas tension.

(3) The manifestation of the hemostatic changes were blocked by the administration of antithrombotic medication in both animals and human divers.

(4) Blocking of the hemostatic mechanism changes with antithrombotic medication did not prevent the occurence of osteonecrosis.

(5) Tissue injury occurred with the first decompression insult and was aggravated by repeated exposure.

(6) Severe endothelial and small vascular damage occurred following compression/decompression exposures which did not produce signs of decompression sickness.

Other data we accumulated suggested certain pathological events or relationships but insufficient information was available for confirmation. They suggest that:

(1) Bubbles passing through a vessel interact with the endothelium to produce the changes that were seen.

(2) Antithrombotic medication facilitates the production of decompression-related osteonecrosis.

(3) Exposure of basement membranes collagen is instrumental in producing hemostatic changes.

(4) Collagen crosslinking is different in animals exposed to inadequate decompression than in normal controls.

I. Radiology

A colony of miniature swine was exposed daily in a hyperbaric chamber, initially to 50 feet for 6 hours, then to 60 feet for 6 hours. In the first profile, the pigs were stage-decompressed at a rate of 20 ft/min. (fpm), to ten feet, then to the surface at 1 ft/min. In a revised profile, the animals were on a continuous decompression at a rate of 30 fpm. Control radiographs were taken of each animal prior to its first exposure and were compared to monthly follow-up radiographs.

Radiographs taken periodically throughout the course of one pig's diving career demonstrated a small suspicious area (Figure 1) in one femoral shaft two and one half months after she was first compressed. Five months later, follow-up radiographs (Figure 2) showed progression in the radiolucency and the appearance of a second radiolucency in the other proximal femoral shaft. Nine months after her initial dive, radiographs showed bilateral mixed radiolucent and sclerotic irregularities (Figure 3), thought to be bone infarcts. Films of the upper extremities (Figure 4) then revealed sclerotic irregular deformities in the proximal humeral shafts. Two months later, films revealed still further progression in the sclerotic bony infarcts.

FIGURE I Small suspicious radiolucency was picked up two and one half months after this miniature pig was first exposed to compression/decompression stress. Arrow points to an irregularly marginated lucency in the subtrochanteric area of left femur.

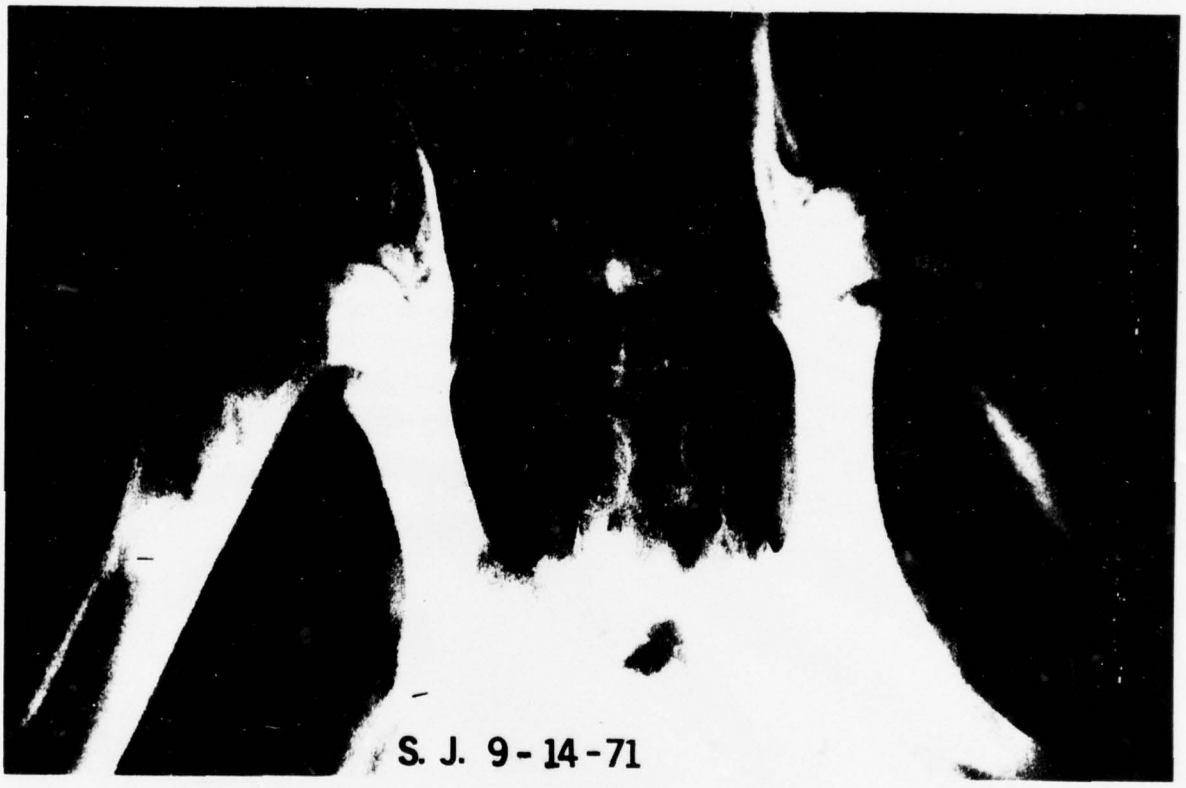


FIGURE 2 Progression in the lesion in the left femur as well as the appearance of a second bilateral lesion was seen in this follow-up film taken five months after the one seen in Figure I.

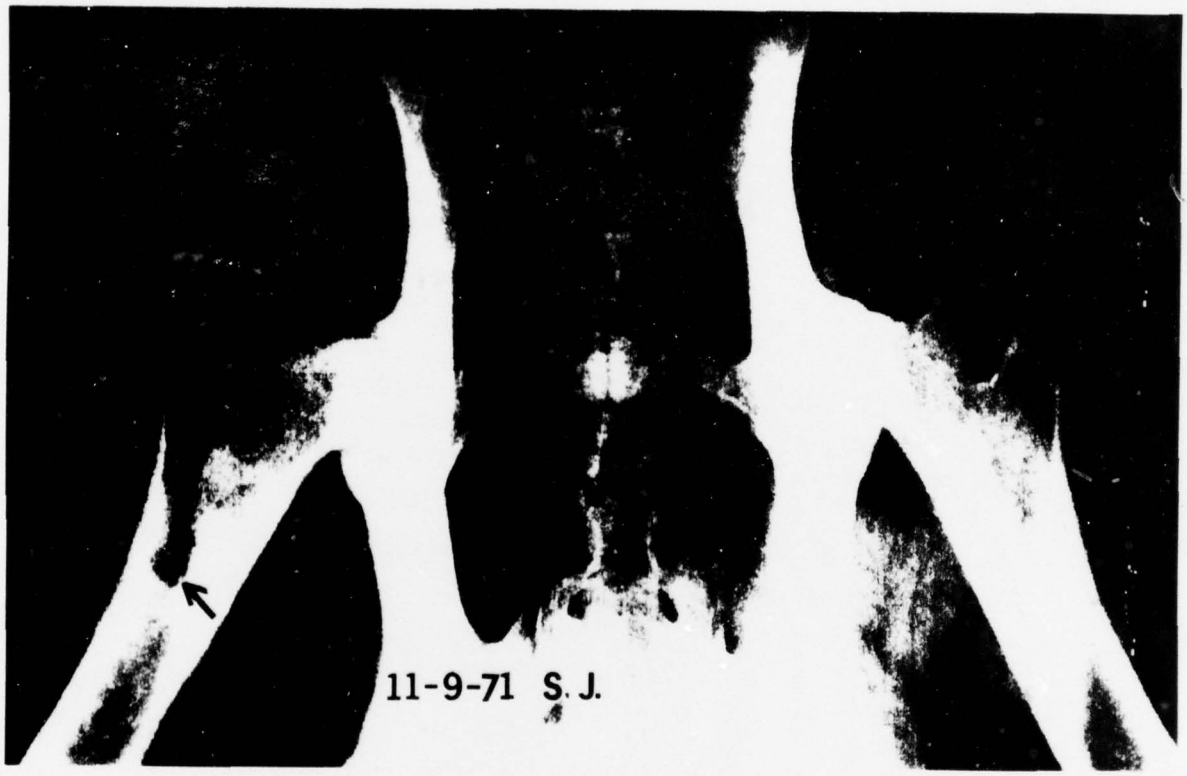


FIGURE 3 Further progression in the sclerotic bony infarcts is seen seven months later as well as the biopsy site (arrow) done two months earlier.


FIGURE 4 Consistent with the necrosis developing bilaterally in the femurs, irregular sclerotic deformities were seen in the proximal humeri.

Thirteen pigs were exposed to multiple rapid decompression episodes from 60 fsw for 6 hours at 30 fpm (Table I); seven others were either terminated to obtain histologic confirmation of bone changes seen radiographically, or died of decompression sickness or related illness while under study. Table I tabulates the findings in these animals. Ten of the group had or have radiographic findings of increased opacity or lucency, sclerotic bands, trabecular distortion, and/or cortical thickening. Two animals died of decompression sickness during the early phases of the study, presumably before the disease could develop, and a third animal remained asymptomatic.

Part I

TABLE I

RESULTS OF MULTIPLE EXPOSURE STUDIES IN 13 PIGS

| PIG# | #DIVES | PROFILE | STATUS | X-RAY | RADIOLOGIC REPORT | HISTOLOGIC REPORT |
|---|---|---|---|---|---|---|
| 1 | 79 | 60ft/6hrs | holding | positive | bilateral mixed sclerotic and lucent pattern 2 months post-initial dive; dense striated bony traveculae developed bilaterally 4 months post-initial dive; humeral, then femoral lesions cleared following 6 month rest. | No histology done |
| 2 | 78 | 60ft/6hrs | holding | positive | sclerotic band seen l. humerus 7 months post-initial dive; no femoral lesions. | No histology done |
| 4 | 48 | 60ft/6hrs | termin. | negative | | microscopic study found areas of bone and fat necrosis not seen by x-ray. |
| 22 | 3<br>1 | 60ft/6hrs<br>100ft/4hrs | holding | positive | areas of increased opacity seen bilaterally 11 months post-initial dive. | No histology done |
| 40 | 40 | 60ft/6hrs | holding | positive | sclerotic streaks in l. humerus developed in 3 months post-initial dive; numerous sclerotic bands developed bilaterally in femurs 4 months after initial dive. | No histology done |
| 58 | 35 | 60ft/6hrs | termin. | positive | Mixed sclerotic and lucent areas developed bilaterally in proximal femoral shaft 6 weeks post-initial dive. | Microscopic study found fibrotic marrow necrotic bone and cortical thickening. |

Part II

TABLE I

RESULTS OF MULTIPLE EXPOSURE STUDIES IN 13 PIGS

| PIG# | #DIVES | PROFILE | STATUS | X-RAY | RADIOLOGIC REPORT | HISTOLOGIC REPORT |
|---|---|---|---|---|---|---|
| 100 | 14 | 60ft/6hrs | termin. | negative | | microscopic study found fibrotic marrow, normal bone |
| 200 | 35 | 60ft/6hrs | termin. | positive | Sclerotic deformities, trabecular distortion and periosteal reaction bilaterally in femoral shafts 5 months post-initial dive; humeral lesions noted 9 months following initial dive. | Biopsy confirmed bone necrosis was present; post-mortem microscopic study found marrow fibrosis as well as additional areas of bone necrosis. |
| 201 | 19 | 60ft/6hrs | termin. | positive | Mixed sclerotic and lucent areas in the femurs bilaterally. | Microscopic study found fat necrosis and endosteal proliferation. |
| 202 | 19 | 60ft/6hrs | termin. | positive | Sclerotic bands bilaterally in femurs 4 months following initial dive. | Microscopic study found marrow fibrosis and areas of bone necrosis. |
| 253 | 25 | 60ft/6hrs | holding | negative | | None |
| 254 | 25 | 60ft/6hrs | termin. | positive | Cortical thickening 5 months following initial dive. | Microscopic study found areas of necrotic bone adjacent to endosteal proliferation; fat necrosis. |
| 80 | 70 | 60ft/6hrs | Holding | positive | Several lucent areas in intertrochanteric femoral shafts associated with bony sclerosis 12 months following initial dive; mottled radiolucencies developed in l. humerus 11 months following initial dive. | None |

Radiography continued to be an important means of monitoring the effects of diving on the long bones of experimental animals. All animals were routinely surveyed prior to exposure, and these films were compared to those taken weekly during the diving series and biweekly thereafter for the early detection of bone changes. Since studies induced osteonecrosis seen radiographically within three weeks of the initial dive on certain profiles (Fig. 5), close observation was necessary to pinpoint as closely as possible the time of onset and allow for histologic examination of bone specimens in the early stages of the development in order to accurately identify etiology of the disease. Long bones were retrieved from all sacrificed animals and radiographed postmortem to aid in the sectioning of specimens for histologic work-up and for comparison with in vivo films. The following reports were selected at random from the file of miniature pig radiographs:

Pig #103

11/27/73 There is a mixed sclerotic and lucent pattern to the bony trabecular detail in the proximal shaft of the left and right femur that has developed since the control films of 10/12/73. This is consistent with osteonecrosis.

01/10/74 There is a changing pattern of mixed sclerotic bands and lucencies in the proximal femoral shafts consistent with an evolving pattern of bony infarct.

02/14/74 There is now a progression in sclerotic areas in the humeral shafts since the study of 01/10/74, and this is felt to be continued evidence of avascular necrosis.

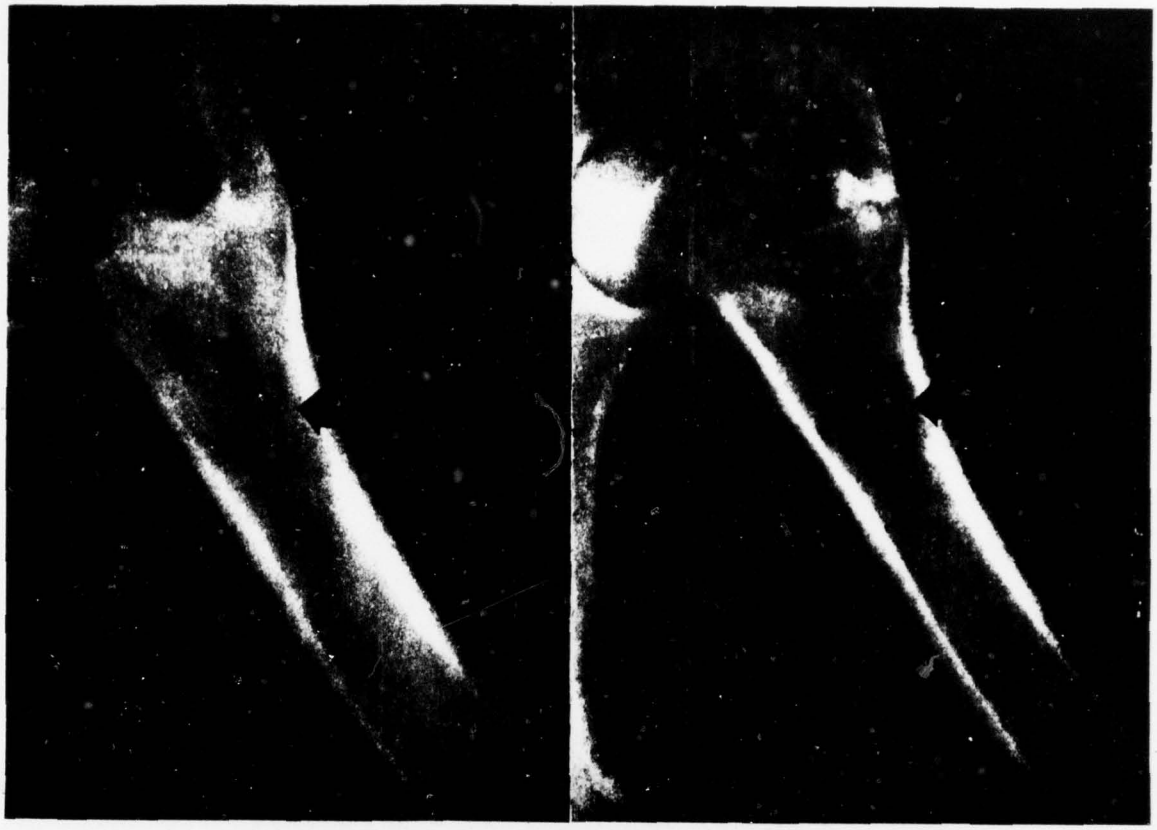

Figure 5. The radiograph of a pig taken after 10 exposures to 60 fsw for 6 hours with 30fpm decompression (a) contrasted with the control radiograph taken before diving (b). The patchy sclerotic and radiolucent areas (arrow, a) developed within 3 weeks of the initial dive in a study designed to evaluate the effectiveness of antithrombotic agents in preventing osteonecrosis. The animal was receiving a combined medication of a platelet function inhibitor, asprin and anticoagulant.

Pig #104
11/26/73 There are numerous sclerotic foci developing in the proximal femoral shafts that were not seen in the control films of 10/06/73. Several sclerotic bands in the proximal shafts of the humeri have also developed since the control study. These findings are consistent with avascular necrosis.

11/26/73 Post-mortem specimens show a mixed sclerotic and lucent pattern to the trabecular body detail in the proximal shafts of the humeri and femurs consistent with osteonecrosis.

Pig #131
03/18/74 The pelvis shows several sclerotic foci in the intertrochanteric area of the left and right femur with proximal shaft lucencies consistent with early osteonecrosis.

04/29/74 A changing pattern of mixed sclerotic and radiolucent areas is seen in the proximal shaft of the left and right femurs. These probably represent a progression in the osteonecrosis seen in the films of 02/18/74.

Pig #152
02/22/74 The pelvis film shows several sclerotic foci and central lucencies in the proximal shaft of the left and right femurs that were not present on the control films of 10/06/73. These are probably bony infarcts secondary to avascular necrosis.

04/29/74 A progression in the sclerotic pattern of both femurs is now seen. Sclerotic bands in the left and right humeri have now developed since the last study. This is consistent iwth osteonecrosis.

Less than 15% of bony changes occurring in experimental animals in this study were undected by x-ray. Occasionally, however, even an advanced case of osteonecrosis did not manifest itself radiographically. In addition to extensive necrosis of the cortical bone with periosteal new bone formation and fibrosis of metaphyseal marrow cavity, attenuation of articular cartilage and thinning of the bone plate was found histologically in one pig whose radiographs appeared to be normal over a period of 18 months.

Osteonecrosis Following Single Decompression Episode

Nine animals exposed to inadequate decompression from a single 60 fsw dive of 6 hours were held from 14 to 18 months for radiographic study of bone changes. Two of these pigs developed evidence of osteonecrosis 10 to 12 months following their single exposure, and a subsequent histologic study confirmed the presence of cellular abnormalities. Two other pigs in the group were chosen at random for sacrifice. Microscopic study of bone specimens revealed in both old marrow fibrosis with some evidence of repair to trabecular bone. Disrupted cement line patterns were noted as evidence of trauma.

Four of the remaining animals without radiographic bone changes were re-dived on the same profile and serially sacrificed within hours of decompression to observe acute bone changes occurring immediately post-dive.

The results of this study confirm the conclusion that osteonecrosis is a sequel to a series of events which begins with the first inadequate decompression.

II Hematology

A. Introduction:

Divers, astronauts, caisson workers and occasionally patients and hospital staff experience changes of ambient pressure. The physiological and biochemical adjustments, associated with pressure alterations are sometimes serious. If decompression is not carried out in a controlled manner, one or more of a wide range of symptoms (decompression sickness) may be produced. Presentation may be sudden with dysfunction of vital organs or delayed with only localized discomfort. Long term sequelae may include auditory loss, vestibular disorientation, paraplegia or aseptic bone necrosis (1).

Proposed mechanisms include bubble formation, fat embolization or blood sludging leading to stasis, infarction and disseminated intravascular coagulation (2,3,4,5). The association between the frequency and intensity of hyperbaric exposures, the decompression schedule, the inert gas employed, bubble formation or other variables and the development of decompression sickness and end organ dysfunction have not been defined. Since the pathogenesis of these complications is not understood, no rational therapy is available to prevent or reverse these sequelae, other than cessation of diving.

Previous studies in this laboratory have demonstrated that miniature swine repeatedly exposed to pressures equivalent to 60 feet of sea water (fsw) for six hours daily with decompression at 30 feet per minute (fpm) have histologic or radiographic signs of osteonecrosis within three to twenty-four weeks of the initial decompression (6,7). Histologic study of these cases of osteonecrosis, as well as evidence presented by other investigators (8,9,10,11) has indicated that

occlusion of blood vessels supplying bone are seen after hyperbaric exposure. The current investigations were designed to determine by kinetic evaluation of the hemostatic mechanism and direct histologic study of blood vessels, the pathologic mechanisms underlying osteonecrosis. Variables of the diving profile were examined for their effects on the hemostatic mechanism in both man and swine and the potential of antithrombotic therapy to modify thrombotic consumption of platelets and fibrinogen was evaluated.

B. Methods:

1. Subjects

A. Hyperbaric exposures in man

All of the subjects participating in these experiments were experienced divers who gave signed informed consent. None of the subjects had dived for at least 48 hours prior to the experimental simulated dive. They had been asked to refrain from consuming alcohol for at least 24 hours prior to the exposure and from taking any type of medication for five days before the dive. The experiments were carried out in a three-lock, high-pressure chamber system (ref.).

Autologous platelets were radiolabeled on the day prior to diving and donor $^{131}I$ fibrinogen and $^{125}I$ plasminogen were injected simultaneously with the $^{51}Cr$ platelets. One blood sample for hemostatic measurements was obtained within one hour post-injection, immediately before diving and after decompression. Samples were then obtained at least daily over the next five days. In the 700 foot dive with decompression over several days bubble free blood samples were brought up from depth (12).

In these subjects, signs of decompression sickness were limited to localized pain or numbness which was alleviated by immediate recompression. Mild itching of the skin was not considered a frank sign of decompression sickness.

United States Navy Standard Air, Royal Navy or Exceptional Exposure Air decompression tables were used for all simulated air dives (13). All mixed-gas dive decompressions were calculated using the Workman mathematical model with Smith-White Mark III ascent criteria and allowable super-saturation ratios (14).

Gas emboli (bubbles) in the blood stream were detected with a Doppler ultrasonic bubble detector as large acoustical discontinuities (15,16). The pulmonary artery was selected for monitoring because of the confluence of venous return at this site and hence it contained the cumulative gas emboli entering the blood stream from the entire body. This artery was monitored by placing the detector transducer in the third intercostal space to the left of the sternal border. Divers were monitored for bubble sounds immediately prior to the dive, during the time spent at depth, throughout the decompression and usually for at lease one hour following the dive.

B. Hyperbaric exposures in swine

The animals studies were Hormel Institute Pig-Mee-Pigs, two or more years old and weighing 50-75 kg. The facility used for the simulated pig dives was a two-lock horizontally oriented hyperbaric chamber, 16 feet long by 6.6 feet wide. The animals were routinely compressed with air at a rate of 60 fpm to 80 fsw for 4 hours or 60 fsw for 6 hours and decompressed at 30 fpm.

In some studies other diving conditions were used to evaluate the resultant effect on hemostasis. To determine the effect of decompression rate, extended staged decompressions of 105 and 180 minutes were also used. Because compression in air to 80 fsw increases the oxygen partial pressure to 544 mmHg, an isobaric chamber exposure was made with a normobaric $pO_2$ to determine the effect of the increased $pO_2$. In addition, some swine were given 100 percent oxygen by mask without diving for 4 hours corresponding to the usual duration of the hyperbaric exposure.

The effects of intravascular bubbles without decompression were determined by infusing 100 to 200 micron bubbles into the venous system of three pigs over a 4 hour period through a small catheter which was passed beyond the surgically implanted jugular catheter. A 50 cc syringe in a Harvard Peristaltic Pump was used to continuously infuse 6 cc of air per minute through the catheter into animals for two hours, the amount was increased to 12 cc per minute at 3 hours, reduced to 6 cc per minute at 3-1/2 hours and to 3 cc per minute at 4 hours before it was discontinued.

For these swine studies autologous platelets were labeled with radiochromium on the day prior to diving and injected simultaneously with labeled donor fibrinogen. Samples were obtained, either by venipuncture or by means of a surgically implanted jugular catheter, within one hour post-injection, immediately pre- and post-dive, twice daily for the next two days and then once on the third post-dive day. In the repetitively dived 60 fsw for 6 hour animals the same weekly labeling and sampling schedule was followed except that blood for labeling was drawn before the animals were dived for the first time that

week and re-injected after surfacing. The next dive was two days after labeling, the last five days after labeling and then the labeling, diving, and sampling were repeated the following week. Hemostatic measurements performed by venipuncture without diving before and after the insertion of a jugular venous catheter showed there were no kinetic changes produced by catheter placement. All the animals were either used for a single study or before a subsequent experiment was performed it was documented that hemostatic values were back to baseline levels. Animals subjected to histologic examination had been exposed only to the dive profile being evaluated.

The criteria for diagnosing decompression sickness in the swine were: 1) signs of joint discomfort as evidenced by leg lifting, weight shifting, refusal to stand; 2) respiratory problems (hyperpnea, dyspnea); 3) CNS disturbance as evidenced by ataxia, stiffness or paralysis, or no response to sensory stimulus and 4) excessive skin petechia (skin bends). Animals with severe bends were recompressed to 60 fsw and then decompressed in stages over 3-4 hours.

Antithrombotic treatment consisted of inhibiting platelet function with: 1) oral sudoxicam (10 mgm per kg/day); 2) oral SH117* (3 mgm per kg/day); or 3) a combination of aspirin (900 mgm per day) and dipyridamole (10 mgm per kg/day) given together orally in three divided doses. In some studies anticoagulation using heparin (every 4-6 hours) or warfarin was used alone or in combination with antiplatelet agents. Heparin was monitored by the recalcification time (17) to keep the whole blood clotting time at twice the control value. Warfarin anticoagulation was regulated to maintain the prothrombin time at 20-25% of baseline values. When studied, antithrombotic therapy was initiated

two days prior to diving and continued for the duration of the kinetic studies.

2. Hemostatic Measurements

Platelet counts were measured with an electronic particle counter on peripheral blood collected in EDTA using the method of Bull et al (18). The platelet count of 100 normal subjects was 250,000/ul ± 40,000 (± 1 S. D.**) and in 19 swine was 329,000/ul ± 119,000. Platelet counting results were the same using phase microscopy (19) compared to Coulter methods in both man and the swine. Platelet survival was determined from the disappearance of radioactivity in blood samples obtained on five to ten different occasions over five to eight days following the injection of $^{51}Cr$ labeled autologous platelets (20). Platelet disappearance curves were analyzed by least squares computer fitting prodcedure using a gamma function as described by Murphy (21,22). Platelet survival in 20 normal subjects was 9.5 days ± 0.6 and in 19 normal swine was 5.2 days ± 0.6. The proportion of labeled platelets remaining in the systemic circulation after infusion (that is "recovery") was calculated from the platelet activity per milliliter extrapolated to zero time, multiplied by the estimated blood volume, and divided by the platelet $^{51}Cr$ activity injected. The estimated blood volume was calculated in man assuming a blood volume of 75 ml per kilogram of body weight and in the swine at 7 percent of body weight (ref.). Platelet recovery in the 20 normal subjects was 65% ± 4 and in

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* SH117 is an experimental drug kindly supplied by Boehringer, LTD., Germany

** Normal values in the Methods section are given as ± 1 Standard Deviation (S.D.)

the swine was 66% ± 19. Platelet turnover per microliter per day was calculated from the peripheral platelet count divided by the platelet survival time in days and corrected for recovery. Expected recovery as found in splenectomized patients was 90%, and any lesser value is assumed to be due to splenic pooling. The recovery factor to correct platelet turnover for pooling in the spleen was $\frac{90\%}{\text{extrapolated percent recovery}}$. Platelet turnover in normal subjects was 35,000 platelets/ul/day ± 4,000 and in the swine was 88,000 platelets/ul /day ± 37,000.

The concentration of fibrinogen was estimated by a modification of the spectrophotometric method of Jacobsson, involving the optical density measurement of thrombin clottable protein after collection on a glass rod and subsequent solution in alkaline urea (23). Fibrinogen concentration in 87 normal nonhospitalized subjects was 2.95 mg/ml ± 0.40 and in the 19 normal swine was 4.37 mg/ml ± 0.83. Fibrinogen labeling was was carried out with either $^{131}I$ or $^{125}I$ after the method of Takeda (24). This technique involves repeated precipitation of fibrinogen with 25% saturated ammonium sulfate followed by solution in 0.05 M sodium citrate, labeling with $^{125}ICl$ or $^{131}ICl$, removal of unbound $^{125}I$ or $^{131}I$ and sterilization by filtration. The solution is maintained at 4°C for one hour followed by centrifugation at 20,000 g for 30 minutes at 4°C in order to remove any cryoprecipitable material. For survival measurements, labeled fibrinogen was prepared each week in multidose amounts from a known hepatitis-free human donor and from a single swine. Comparative survival studies were made in six normal subjects, using both donor and autologous fibrinogen before and after storage at 4°C for one week. The results showed no effect of storage or

fibrinogen source on the rate of disappearance. Initial survival was 5.1 days ± 0.2 compared with a survival of 5.1 days ± 0.3 following seven days storage at $4^{o}C$. Fibrinogen survival in normal swine was 3.6 days ± 0.5. Bacterial cultures, made after the fibrinogen solutions were stored at $4^{o}C$ for one week, consistently showed no growth. Fibrinogen survival was determined from the half-time disappearance divided by the natural logarithm of 2. Fibrinogen turnover was used as a measure of fibrinogen destruction, and was calculated from the fibrinogen concentration divided by the mean survival time. The value is 0.57 mg/ml/day ± 0.06 in normal subjects and 1.23 mg/ml/day ± 0.30 in the swine. Oral doses of nonradioactive iodine (5 MEq of KI twice daily) were begun the day before injection of radioiodine labeled fibrinogen in the human studies and continued for 10 days.

Plasminogen levels were determined by a caseinolytic (25) technique in the human studies and are expressed as micrograms per milliliter of plasma, calculated on the basis of a specific activity of 25 CTA units per milligram of plasminogen (25). Concentration averaged 105 mg/ml ± 20 in 65 normal subjects (equivalent to 2.63 CTA units per ml ± 0.51) (27). Plasminogen for $^{125}I$ labeling was prepared from single donor plasma by one-step EACA-agarose affinity chromatography as described by Deutsch and Mertz (28). Two important modifications of this method were necessary for reliable in vivo results: 1) all purification procedures were carried out at $4^{o}C$, and 2) saturation binding of plasminogen (achieved by using excess plasma) was used to displace non-specifically bound protein from the column during loading. The resulting plasminogen preparation was biochemically homogenous as assessed by SDS poly-acrylamide gel electrophoresis, immuno-electrophoresis, cellulose

acetate electrophoresis and N-terminal analysis, with specific activity of 25 CTA units per mg of protein (ninhydrin) and a calculated molecular weight of 85,000. Chloramine T was used for $^{125}I$ labeling (29). In vivo plasminogen survival was determined by dividing the T 1/2 disappearance time of labeled plasminogen by the natural logarithm of 2. In 20 normal subjects, plasminogen had a logarithmic disappearance curve with a mean survival of 2.3 days $\pm$ 0.2. Plasminogen turnover (concentration/survival), an estimation of the rate of in vivo consumption, was regarded as reflecting overall fibrinolytic activity in vivo. The 20 normal subjects had a mean plasminogen turnover of 45 ug/ml/day $\pm$ 5. Plasminogen kinetic measurements were not performed in the swine. For all diving studies in both humans and swine the labeled hemostatic factors were mixed before injection so that their introduction was simultaneous.

The concentration of factors II, V, VII/X, VIII, IX and X were performed in these studies by standard methods (30). In addition, fibrinolysis was also assessed by measuring fibrinogen/fibrin related antigen (F-FRA) levels using a hemagglutination inhibition technique as described by Mersky (31) from samples prepared with thrombin/EACA at $4^{o}C$ (32). In 56 normal subjects, this measurement was consistently less than 0.5 ug/ml.

III. *Histologic Techniques*

Vessels were obtained for histologic examination in 18 anesthetized animals: 9 controls and 9 dived repetitively (60 feet for 6 hours with decompression at 30 feet per minute, 3 times a week) for three months.

Vessel Morphological Procedures

Three types of vessel preparation were obtained: 1) lower abdominal aorta, iliac and femoral arteries for light and electron microscopic

examination of lesion formation; 2) carotid arteries for $^{3}$H-thymidine incorporation by endothelial cells; 3) thoracic and upper abdominal aorta for silver stain quantitation of endothelial coverage. Specimens were obtained under general (halothane) anesthesia and assisted respiration in the following way. After opening the abdomen, the aorta and inferior vena cava were carefully freed up by sharp dissection without affecting flow. The inferior vena cava was then cannulated to establish isolated venous drainage from the lower limbs and immediately thereafter the aorta was cannulated, flushed free of blood elements with 5% glucose solution at 100 mm Hg of hydrostatic pressure, and perfuse-fixed with buffered half-strength Karnovsky's fixative under 100 mm Hg of pressure for 20 minutes in vivo. Once perfusion fixation of the distal arteries was underway, the carotid arteries were freed up by sharp dissection, isolated, and carefully removed. The artery segments were gently flushed free of blood with saline, and excess adventitia removed for $^{3}$H-thymidine studies. Immediately after removing the carotid arteries, the chest was entered (thereby interrupting respiration), the arch of the aorta quickly cannulated and drainage outflow was established above the renal arteries. The aorta was then cleared of blood using 5% glucose solution at 100 mm Hg pressure. In vivo fixation was then carried out by perfusion-fixation of the thoracic and upper abdominal aorta with 0.3% silver nitrate solution at a pressure of 100 mm Hg for 20 minutes. After perfusion-fixation, the vessels were removed by sharp dissection. The foregoing procedure ensured the procurement of three different artery preparations from a single living animal while using isolated regional pressure-perfusion fixation.

At the time of sacrifice the aorta was cannulated below the renal vessels flushed free of blood with 5% glucose solution and then perfuse-fixed with buffered half strength Karnovsky's fixative (28) and under 100 mm Hg of hydrostatic pressure for 20 minutes in vivo. Vessels were subsequently removed by sharp dissection and placed in half strength Karnovsky's fixative for 6 hours at $4^{o}C$. Segments from abdominal aorta, and each iliac artery were removed for scanning EM. The remaining vascular tissues were subsequently cut into approximately 1 mm rings and returned to the fixative for another hour at $4^{o}C$. They were then washed in 0.1 M cacodylate buffer containing 0.2 M sucrose (pH 7.3) and subsequently post-fixed in 1% oxmium tetroxide buffered with s-collidine or with phosphate buffer (pH 7.3) for 1½ hours, followed by enblock staining with 2% uranyl acetate for ½ hour. After embedding in epoxy resin, thick sections were cut at 1 micron as circles, and stained with a combination of basic fuchsin and azure II-methylene blue for light microscopy. Selected thin sections were cut at approximately 800 Å thickness for transmission electron microscopy. These sections were stained with lead citrate followed by uranyl acetate. All of the sections for electron microscopy were examined in an AEI801 electron microscope.

$^{3}$H-thymidine Labeling Index (TLI)

Both carotid arteries were removed by sharp dissection, flushed with saline and cut into 20 mm lengths. Whole segments were placed in 3-4 ml of medium TC 199 containing 1 uc $^{3}$H-thymidine/ml and incubated at $37^{o}C$ for 4 hours. The vessel segments were removed, trimmed, opened longitudinally and mounted flat for fixing 24 hours in 10% neutral buffered formaldehyde. The endothelium was prepared for autoradiography

by the following method (ref.). The luminal surface of the specimen was embedded onto a slide in collodion leaving the adventitial surface exposed. The adventitial surface was then embedded in gelatin and bonded to a gelatin-coated slide by fixation with formaldehyde while the specimen and the coated slide were held together under pressure. Subsequently the collodion overlying the endothelial cells was removed with ether and alcohol thereby exposing the luminal surface of endothelial cells for coating with autoradiographic emulsion. Autoradiography was performed by hand dipping endothelial mounts in Kodak NTB-2 emulsion, 2 weeks exposure, followed by Kodak D-19 developer and rapid fixer. The preparations were stained with Harris' hematoxylin and mounted.

About 80% of the mounts were complete, containing over 90% of the endothelial cells intact and countable. All of the endothelial cells from each mount, i.e., 15,000 cells each were scored as labeled or unlabeled and the $^{3}$H-thymidine labeling index (TLI) represented the ratio of these values expressed as a percentage.

Endothelial Cell Loss (ECL)

Pressure-perfusion fixation was carried out of the thoracic and upper abdominal aorta with 0.3% silver nitrate solution at the time of sacrifice in vivo (33). The entire endovascular surface of whole-aorta mounts were then examined morphometrically by means of a grid micrometer, and the proportion of the surface that was not covered by endothelial cells was determined.

Multiple tissue sections were prepared by conventional microscopy from heart and both femurs, kidneys and lungs for a quantitative analysis of infarction and vascular changes.

C. Results

1. Human Studies

A. Kinetic evaluation of hemostasis

Platelet, fibrinogen and plasminogen kinetic data following 9 different dive profiles in 53 studies in male divers are shown in Table I. Serial changes in the concentration of platelets, fibrinogen, plasminogen, factors II, V, VII, VII/X, VIII, IX, XI and the hemaglutination inhibition test for fibrinogen degradation products were also followed before and after some of the dive profiles. In the majority of dives, listed in increasing order of depth, compressed air was used and decompression was by U.S. Navy tables.

The first dive of 100 fsw for 60 minutes produced no significant abnormalities in platelet or fibrinogen kinetic data in any of the four subjects compared to controls. Decompression was by the British Royal Navy tables. Platelet counts were decreased to an average of 93% $\pm$ 4* of baseline on the first post-dive day, 93% $\pm$ 3 on the second and were normal by the third day (100% $\pm$ 3) (Table II). Fibrinogen concentrations increased slightly to 105% $\pm$ 8 of baseline on the first post-dive day and were normal thereafter. There was no evidence of bubbles by Doppler measurements or decompression sickness in any of these subjects.

To determine if this profile would produce kinetic changes if performed repetitively (as would occur for example with caisson workers), 4 divers were dived daily on this profile for 14 days with hemostatic kinetics repeated twice a week for a total of 4 studies each. There

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* Variances in the Results section are given as $\pm$ 1 Standard Error ($\pm$ 1 S.E.)

was a progressive shortening of the platelet survival to a low of 4.4 days ± 0.2 after 10 dives, then some improvement towards normal after 14 dives (5.5 days ± 0.7) (Figure 1 and Table I). Platelet turnover remained consistently elevated at 1.6 x normal from the third through the fourteenth dive (Table I). Fibrinogen survivals were modestly shortened for the first 3 dives and then returned towards normal with no significant changes in fibrinogen turnover (Figure 1 and Table I).

After sequential dives of 150 fsw for 10 and 15 minutes on two subsequent days in five subjects platelet survival was reduced to 6.7 days ± 0.6 ($p <$ ) and turnover was 48,000 platelets/ul/day ± 9,000 (1.4 x normal ± 0.3, $p <$ ). Fibrinogen kinetics were normal in all five as were plasminogen kinetics in the two who were studied (Table I). Platelet counts were 103% of baseline ± 3 on the first post-dive day, 96% ± 5 on day two, and 99% ± 4 on day three. Fibrinogen concentration was 103% ± 5 of baseline on day one, 107% ± 6 on day two and 110% ± 4 on day three (Table II). None of the subjects had decompression sickness but it is of interest to note that all three subjects with documented bubbles in their pulmonary arteries during the course of diving had transient platelet sequestration for periods up to 48 hours after diving. Shortened platelet survivals appeared to occur independently of detectable bubbles since one dive with detectable bubbles had a platelet survival of 5.3 days and two with bubbles had normal platelet survivals; the converse of decreased platelet survivals without detectable bubbles was also documented.

Following a single dive of 200 feet for 60 minutes in four subjects platelet survival was reduced to 7.0 days ± 0.8 ($p <$ ). Fibrinogen

survivals and turnovers were not significantly different from normal and averaged 4.6 days ± 0.3 and 0.61 mgm/ml/day ± 0.02, respectively. Plasminogen kinetics were normal (Table I). Platelet counts were unchanged and fibrinogen concentration increased on the first post-dive day to 106% ± 2 of baseline and was normal by the next day (Table II).

Following a dive of 220 fsw for 20 minutes in 12 subjects platelet survival was reduced to 6.4 days ± 0.4 ($p <$ ); and a turnover of 53,000 platelets/ul/day ($p <$ ). Platelet recovery was increased to 78% ± 4 ($p <$ ). Fibrinogen survival averaged 4.3 days ± 0.2 and turnover was not significantly different from normal at 0.59 mgm/ml/day ± 0.04. Plasminogen survivals averaged of 1.8 ± 0.2 with a turnover of 68 ug/ml/day ± 11 ($p <$ ). Four of these divers had repeat kinetic measurements one week post-dive, and survival times were consistently longer (Table I, $p <$ ). Bubble formation occurred in three divers and decompression sickness requiring recompression in two of these. Platelet counts were 97% ± 3 on day one and had increased to 103 ± 3 on day three post-dive. Fibrinogen concentrations were 96 to 98% of baseline in the three days post-dive (Table II).

The remaining dives were to 300 fsw for 30 minutes and 400, 500 and 700 fsw for 60 minutes each. All of these dives were with a mixed gas (Praeger). Seven of the 8 divers had evidence of bubble formation and one of the 700 foot divers had unilateral transient deafness as the only manifestation of decompression sickness. The 300 foot dive was associated with normal platelet kinetics and a shortened average fibrinogen survival of 4.1 ± 0.1 days without a significant increase in turnover (0.78 mgm/ml/day ± 0.16 which is 1.3 x normal ± 0.2). Plasminogen survival was also decreased to 1.6 x 0.1 days and turnover was 90

ug/ml/day ± 1 (2.0 x normal) (Table 1). Platelet counts had a maximum depression to 93% ± 2 of baseline on day two and were 104% ± 4 on day three. Fibrinogen concentration was unchanged (Table II).

All five of the individuals who dived beyond 300 fsw had shortened platelet survival times with means of 5.9 days, 5.2 days and 2.9 days and turnovers of 70,000, 73,000 and 107,000 platelets/ul/day for 800, 500, 700 fsw, respectively. Platelet counts were variable for this group of dives in the post-dive period (Table III). Fibrinogen survivals and turnovers were normal in the divers at 400 and 500 fsw but shortened to 3.3 days ± 0.3 with turnovers of 1.6 x normal in the 700 fsw study. Fibrinogen concentrations were elevated to 125% ± 8 of baseline only on day 1 following the 200 fsw dive (Table II). In the two divers who dived to 400 fsw for 60 minutes plasminogen survival averaged 1.6 ± 0.4 and turnover was increased to 3.2 x normal ± 0.3. Kinetic measurements were improved one week after the 700 fsw dive (Table I).

There appears to be a direct relationship between decreasing platelet survival and increasing depth of diving (dives for 60 minutes with decompression by standard Navy profiles) (Figure 4).

B. Coagulation and fibrinolysis factors

Factors II, V, VII/X, VIII, IX were followed during the 200, 220, 300 and 400 fsw dives, and no significant changes were seen in any

of the studies. Hemaglutination inhibition tests were performed serially in all divers before and after the dives of 100, 200 and 158 fsw for 60 minutes and the 220 fsw dive for 20 minutes and none had values above normal.

C. Bubble detection

In the 16 divers in whom bubbles were detected in circulation, sequestration of platelets for 5 to 56 hours was found in 10 (63%). Five subjects showing bubbles without sequestration had the deepest dives (400, 500 and 700 fsw) and shortest platelet survivals (Table I). In the other 18 divers in whom bubbles were not found only one showed transient sequestration for five hours (6%). However, bubbles were observed in divers both with and without signs of decompression sickness.

D. Oxygen concentration

The effect of oxygen tension on the hemostatic mechanism was determined by a dive of 158 fsw for 60 minutes using 96% nitrogen and 4% oxygen. This was comparable in depth and duration to the dive of 200 fsw for 60 minutes. However, the gas mixture with 4% oxygen maintained normal atmospheric oxygen tension at depth while the compressed gas used with the 200 fsw dive produced increased oxygen tensions at depth.

The four divers on the normoxic profile had shortened platelet survival (6.6 days $\pm$ 1.0, $p <$ ) and a turnover of 61,000 platelets/ul/day $\pm$ 5,000 ($p <$ ). A comparable increase in fibrinogen destruction was also found (3.8 days $\pm$ 0.2, $p <$ and a turnover of 0.76 mgm/ml/day $\pm$ 0.07 $p <$ ). Plasminogen survival was decreased to 1.9 days $\pm$ 0.1 ($p$ ) but turnover was not increased (43.0 $\pm$ 4 ug/ml/day). These values do not differ significantly from the 200 fsw dive for 60

minutes on compressed air (Table III). Serial platelet and fibrinogen concentrations were unchanged from baseline (Table II).

E. Treatment with platelet function inhibitors

Four divers had dives of 220 fsw for 20 minutes on two consecutive days with a repeat study done in the same group of divers one month later while taking 75 mgm dypridamole and 330 mgm of ASA three times daily (Table IV). Platelet survivals averaged 5.2 days ± 0.5 baseline and 7.3 days ± 0.4 on aspirin and dypridamole ($p <$ ). Fibrinogen survivals averaged 4.4 days ± 0.1 and 4.5 days ± 0.2, respectively not different from baseline or from each other (p greater than 0.05).

2. Studies in Miniature Swine

A. Hemostatic kinetics

Two hundred and seventeen kinetic studies were carried out in miniature swine. Baseline values in normal animals were: platelet count 329,000/ul ± 25,000; recovery 66% ± 4; survival 5.2 days ± 0.1 and turnover 88,000 platelets/ul/day ± 10,000; fibrinogen concentration averaged 4.38 mg/ml ± 0.27, survival 3.6 days ± 0.1 and turnover 1.23 mg/ml/day ± 0.08 (Table V).

After a single dive of 80 fsw for four hours followed by decompression at 30 feet/minute in 7 animals the mean platelet count was unchanged at 291,000/$mm^3$ ± 50,000, recovery was increased to 93% ± 5, ($p <$ ) survival was reduced to 2.3 days ± 0.3 ($p <$ ; Figure 5) and turnover was increased (139,000 platelets/ul/day ± 21,000, $p <$ ) (Table V). The platelet count was maintained normal despite decreased survival by mobilizing the splenic pool. Fibrinogen concentration was

increased to 6.28 mg/ml ± 0.52, survival decreased to 2.1 days ± 0.3 and there was a 2 1/2 fold increase in turnover to 3.13 mg/ml/day ± 0.27 (Figure 6). While platelet and fibrinogen survivals were both reduced to about 1/2 normal, fibrinogen turnover exceeded platelet turnover because fibrinogen production was disporportionately elevated (Table V).

By the first post-dive week the platelet count had increased to 383,000/ul ± 46,000 and platelet kinetic measurements were returning towards normal, i.e., recovery 85% ± 10, survival 3.5 day ± 0.4, and platelet turnover 128,000 platelets/ul/day ± 15,000, and Table V). Fibrinogen concentration remained increased at 5.35 mg/ml ± 0.46; fibrinogen survival averaged 3.8 days ± 0.4 and turnover was 1.49 mg/ml/day ± 0.3 which are both within the normal range (Figure 6 and Table V). Fibrinogen concentration remained elevated through the fourth post-dive week. Platelet counts, recovery, survival and turnover gradually became normal by the fourth post-dive week (Figure 5 and Table V). After the immediate post-dive period, platelet survival was selectively shortened compared to fibrinogen. Platelet survival required 4 weeks to return to normal while fibrinogen survival was normal after the first study (Figures 5 and 6). The presence or absence of decompression sickness was not correlated with any specific change in the hemostatic measurements.

Since repetitive dives of 60 fsw for 6 hours have been shown to produce femoral osteonecrosis, kinetic studies were performed using this diving schedule 3 times a week in 6 animals. In these studies it required from 9 to 18 exposures to shorten platelet and fibrinogen survivals to the same degree that they had been following a single dive of 80 feet for 4 hours (Figure 7).

B. Controlled decompression

Instead of rapid uncontrolled ascent from depth at 30 feet/minute, one animal was decompressed from a dive of 80 fsw for 4 hours over 105 minutes in stages and platelet survival was 2.6 days and fibrinogen was 1.9 days in the immediate post-dive period (week 0) (unchanged from uncontrolled ascent) but both were normal by the first post-dive week (5.0 day platelet and 4.5 day fibrinogen survival). These values are closer to data in normal animals at this time without controlled ascent (platelet survival 2.5 days ± 0.4 and fibrinogen 3.8 days ± 0.4). In two animals further normalization resulted when decompression was extended to 180 minutes (one had platelet survival of 5.4 days and fibrinogen survival of 3.5 days in the immediate post-dive period). The other animal showed prolongation in the platelet survival to 3.8 days and fibrinogen to 2.9 days. Platelet and fibrinogen survivals were normal one week after diving.

C. Oxygen concentration

Since breathing 100% oxygen produces endothelial cell injury in the pulmonary circulation (36) and animals dived on compressed air have oxygen tensions exceeding the partial pressure of oxygen at sea level, the effects of changing the oxygen concentration on the kinetics of platelets and fibrinogen were studied. Animals were dived at 80 fsw for 4 hours with rapid decompression at 30 feet/minute while changing the usual compressed air (20% $O_2$, 80% $N_2$ at 80 fsw) to a mixture of 92.8% nitrogen and 7.2% oxygen which at depth would give an equivalent partial pressure of oxygen to atmospheric conditions (4.5 ft. $O_2$, 58.7 ft. $N_2$, etc.). Initially, two animals were given 100% oxygen to breathe by mask for four hours without decompression to see if any hemostatic

changes occurred. The survival measurements in these non-dived animals on oxygen were unchanged from baseline (Table VI).

Animals dived on the normoxic gas mixture showed similar changes in their kinetic studies immediately following the dive (Table VI) as the animals dived with compressed air (Table V). However, six of the seven swine (86%) had symptoms of decompression sickness and three out of the six (50%) had to be recompressed. This compares with decompression sickness in two out of seven (29%) of the animals dived on compressed air, neither of which were severe enough to require recompression. Moreover, only two of the seven normoxic animals had platelet and fibrinogen survivals return to baseline values within the period of nromalization observed in control animals i.e., four weeks post-dive for platelets and one week post-dive for fibrinogen (Table V and VI, Figures 5, 6, 8). Correction of platelet survival time was delayed 6 weeks or longer in the remaining five animals.

D. Intravascular bubbles

Two animals had bubbles infused via an indwelling catheter for 4 hours without hyperbaric exposure to evaluate the effects of bubble formation on hemostasis. Labeled platelets were sequestered outside the general circulation after bubble infusion but returned to the circulating blood pool after 29 and 39 hours. Platelet and fibrinogen survival times were not changed from baseline values.

E. Antithrombotic therapy

To determine if antithrombotic therapy is capable of modifying the increased utilization of hemostatic factors, groups of animals were treated with either platelet function inhibitors (combination of dipy-

ridamole and ASA, sudoxicam, or SH117), anticoagulants (heparin or warfarin), or a combination of platelet function inhibitors and anticoagulation before, during and after the dive profile of 80 fsw for four hours and decompression at 30 feet/minute. In six animals platelet function inhibitors alone showed no effect on any of the hemostatic parameters in the immediate post-dive period (week 0) over the findings in the untreated animals (Tables V and VII). In the 3 animals continued on therapy, however, platelet survival was normal at 5.0 days $\pm$ 0.3 in the first post-dive week compared to 3.5 days $\pm$ 0.2 in the untreated swine (Figure 9A and Tables V and VII). In the one unmedicated animal survival at one week was 3.2 days. During the second post-dive week when all the animals had been taken off therapy the platelet survival shortened to 3.4 days $\pm$ 0.2. Platelet survival returned to normal by the third post-dive week in three of the animals and was still short at the fourth post-dive week in the fourth. Fibrinogen survival, as expected, was normal by the first post-dive week (3.9 days $\pm$ 0.1 treated versus 3.8 days $\pm$ 0.4 untreated) (Figure 9B).

Treatment with anticoagulants (heparin or warfarin) in four animals produced minimal improvement in platelet survival (3.1 days $\pm$ 0.1 compared with 2.3 $\pm$ 0.3 in the untreated animals, p greater than 0.___) in the immediate post-dive period (week 0) and no change in fibrinogen survival (Tables V and VII and Figure 9). Anticoagulation also prolonged platelet survival after the first post-dive week similar to antiplatelet agents, i.e., platelet survival increased to 4.5 days $\pm$ 0.2 compared with 3.5 days $\pm$ 0.2 in the untreated animals (p less than 0).

A combination of both a platelet function inhibitor and an anticoagulant prevented platelet and fibrinogen destruction in the immediate

post-dive period (week 0) in three animals (4.7 days ± 0.4 platelet and 3.5 days ± 0.5 fibrinogen survival compared to the untreated survivals of 2.3 days ± 0.3 and 2.1 days ± 0.3, respectively) (Tables V and VII and Figure 9).

Similarly, although a combination of anticoagulation and platelet function inhibition could protect against the consumption of platelets and fibrinogen in the three chronically dived animals tested (60 fsw for 6 hours, 3 times a week), it did not prevent progression of the injury, as illustrated by platelet survival measurements obtained during alternating intervals with and without combination therapy (ASA, dipyridamole and warfarin) (Figure 7). In the repetitively dived animals platelet survivals averaged 2.8 days ± 0.3 the week prior to starting treatment and 4.5 days ± 0.3 one week after starting combination treatment. Similarly, fibrinogen survivals averaged 2.5 days ± 0.1 before and 3.0 days ± 0.6 after therapy.

F. Vascular effects

The loss of aortic endothelial cells was measured in fifteen animals using silver stained whole aorta mounts after in vivo pressure perfusion fixation. About 10% (range 4-25%) of aortic endothelial surface was absent in 8 animals repeatedly dived (Figure 10, Table 8). Platelets were found to react to exposed subendothelium (Figure 10). Endothelial cell loss was not significantly different in seven treated animals undergoing the same diving schedule while ingesting a pharmacologic inhibitor of platelet function (SH-117 3 mg/kg/day) (Table IV).

In the repeatedly dived animals the medium sized arteries in osseous tissues demonstrated intimal thickening .

D. Discussion

The use of combined platelet and fibrinogen kinetic measurements permits a dynamic evaluation of thrombus formation; i.e. - platelet plug formation (manifested by a decrease in the survival of circulating platelets) with or without associated fibrin deposition (shortened or normal fibrinogen survivals, respectively). When plasminogen kinetics are added, associated fibrinolysis can also be followed. The advantage of a kinetic approach is the ability to sensitively monitor increased utilization of hemostatic factors since their concentration in circulation reflects not only destruction but production rates and changes in storage pools. Therefore, the concentration of a hemostatic factor may remain stable despite significant utilization if compensatory mechanisms are adequate.

These studies in both man and swine have shown increased destruction of platelets and fibrinogen with secondary activation of fibrinolysis following some hyperbaric exposures without finding significant decreases in the concentration of any of the hemostatic factors. In the animal model a dive of 80 fsw with a bottom time of four hours followed by rapid decompression (30 feet/minute) immediately shortens platelet survivals from the normal average of 5.2 days $\pm$ 0.1 ($\pm$ 1 S.E.) to an average of 2.3 days $\pm$ 0.3 and fibrinogen survivals from 3.6 days $\pm$ 0.1 to 2.1 days $\pm$ 0.d. However, the threefold increase in the rate of destruction of platelets and fibrinogen is not reflected in the concentration of these factors in circulation. In fact, the fibrinogen concentration increased from 4.37 mg/ml $\pm$ 0.21 to 6.28 mg/ml $\pm$ 0.52 while the platelet count remained essentially unchanged at 291,000/ul $\pm$ 50,000 vs. 329,000/ul $\pm$ 25,000. However, the marked

increase in labeled platelets recovered in circulation from the normal of 66% ± 4 to 93% ± 5 indicates that platelets, like fibrinogen, have been mobilized from storage pools which obscures the expected fall in concentration secondary to increased destruction. This mobilization of platelets and fibrinogen from storage depots may be mediated by epinepherine as a consequence of the stress associated with diving since physiologic amounts of epinepherine releases platelets from the splenic pool and fibrinogen from liver cells (37). Serial studies after this dive showed that platelet counts one and two weeks post-dive were 383,000/ul ± 46,000 and 328,000/ul ± 45,000, respectively, reflecting the gradual return of platelet recovery to normal, and progressive normalization of platelet survival. Figures 5 and 6 clearly demonstrate the relationship between increased destruction, immediate mobilization from storage pools and concentration of platelets and fibrinogen. However, other investigators have found that platelet counts may be depressed after hyperbaric exposure in man; i.e. - a platelet count of 75% of baseline was found three days after a dive of 100 fsw for 60 minutes and decompression by the British Royal Navy Schedule (38,39). Our divers on this profile showed only minimal reduction in platelet counts i.e., the maximum fall was 93% ± 3 of baseline. Out of the nine dive profiles in man the two deepest produced only slight depresssion of the platelet count (85% of baseline, Table II) and the fibrinogen concentration always showed an immediate increase similar to the studies in the animal model. Findings not unlike the hemostatic response seen following some hyperbaric dive profiles are routinely seen following surgical injury. Surgical stress mobilizes platelets and fibrinogen from storage pools which maintains normal to increased concentrations of

these factors in circulation in spite of their increased utilization in response to the tissue injury (40).

When hemostatic measurements were followed serially at weekly intervals after diving in swine, marked differences were found in the time required for the platelet survival to return to normal (4 weeks) compared to fibrinogen survival (1 week). Since factor consumption was much more modest in man this differential was not apparent as both hemostatic components returned to normal by one week. These data may indicate that two independent mechanisms may be operating to produce hemostatic factor consumption.

In an effort to clairfy the underlying causes of hemostatic factor destruction and their prevention, various combinations of antithrombotic agents were administered in the swine before, during and following the 80 feet for 4 hour dive profile and during repetitive diving at 60 fsw for 6 hours. These studies provided four important observations; 1) immediately following the dive (week 0) the increased utilization of platelets and fibrinogen was prevented only with combination treatment with a platelet function inhibitor (sudoxicam or combined dipyridamole and ASA) and anticoagulants (coumarin or heparin); 2) by the first post-dive week when only platelet survivals remained shortened, either a platelet function inhibitor or an anticoagulant alone were able to prevent platelet consumption; 3) discontinuation of antithrombotic therapy before the expected time for normalization of survivals indicated that the injury which produced hemostatic consumption was not prevented but only its expression since hemostatic survivals become comparably decreased to those of untreated animals, and 4) progressive normalization of platelet and fibrinogen survivals were not inhibited by

antithrombotic therapy. Similar studies in man showed improvement in platelet survivals with the administration of platelet function inhibitors. The requirement for both a platelet function inhibitor and an anticoagulant immediately post-dive to prevent consumption further suggests that platelet plug formation and fibrin deposition may be occurring by more than one mechanism. Previous studies evaluating the response of various thromboembolic states to antithrombotic therapy have shown that in diseases associated with predominant platelet consumption (artificial surfaces in circulation, i.e. - heart valves, A-V renal cannulas and prosthetic aorta-femoral grafts and vasculitis), platelet function inhibitors alone are appropriate treatment; while in diseases associated with combined consumption of platelets and fibrinogen (venous thrombosis), anticoagulants and not platelet function inhibitors are effective therapy (41). Patients with artifical surfaces in circulation and vasculitis probably have two important common factors which produce the isolated platelet destruction; i.e. - a nonendothelialized surface in circulation and rapid arterial flow which prevents significant fibrin formation in the area of the platelet plug. In the context of these previous observations the hemostatic abnormalities seen in the divers may be a combined removal of platelets from circulation by dive related endothelial injury in the microcirculation and, since this is in an area of slow flow, subsequent fibrin formation with further platelet utilization occuring simultaneously. Progressive normalization of platelet survival during the four weeks after diving suggests progressive re-endothelialization of damaged vessels. This type of sequence has been well documented in studies in a baboon model which indicated there was a quantitative relationship between the progressive

re-endothelialization of an artificial surface in circulation (aorta-femoral graft) and normalization of platelet survival (42). The finding of significant endothelial cell loss and platelet consumption in animals repetitively dived indicates that hyperbaric exposure induces endothelial damage which in turn produces platelet consumption, narrowing of the vessel lumen, reduced flow and secondary fibrin formation. Presumably a platelet function inhibitor prevents platelet plug formation in an area of low flow in the immediate post-dive period. Local tissue destruction with release of tissue thromboplastin into circulation may also contribute to fibrinogen and platelet consumption. Although there is predominant platelet consumption after the dive week the fact that either an anticoagulant or a platelet function inhibitor alone is able to prevent platelet destruction suggests that neither pathologic abnormality has been completely resolved; but that, with prevention of at least one of these processes, the other becomes insignificant.

Several previous investigators have suggested that intravascular bubble formation may cause platelet adhesion, aggregation and platelet destruction (43). Although platelet sequestration was positively correlated with intravascular bubble formation in both man and the swine, it is unlikely that these bubbles would remain in circulation for several weeks; indeed these studies have shown that platelets return to the circulation within 56 hours of diving in man and immediately after cessation of bubble infusion in the swine. This indicates that bubbles produce only reversible removal of platelets from circulation and are not the cause of sustained platelet destruction but does not exclude the possibility that they may induce loss of endothelium.

Since not all dives in either man or animal were associated with hemostatic abnormalities the differences between injurious and non-injurious dive profiles were further evaluated. The method of decompression was clearly important since controlled rate decompression following the standard dive profile in the swine of 80 feet for four hours was able to prevent completely the development of hemostatic abnormalities. Previous studies have suggested that the more important physiologic change that occurs with controlled rate decompression is the number of bubbles which are formed in circulation. We therefore conclude that intravascular bubble formation causes vascular injury and endothelial cell loss. Platelet consumption is a secondary consequence of subendothelial exposure to circulating platelets. Further studies in man suggested progressive increases in platelet utilization with depth of diving in spite of apparent adequate decompression schedules.

Another important variable may be tissue oxygenization since, both in man and the animal model, dives under decreased oxygen tensions were associated with a high incidence of decompression sickness and delayed normalization of platelet survival, suggesting more tissue damage.

## SUMMARY

Utilizing labeled platelet and fibrinogen kinetic measurements of hemostasis it has been found that a dive profile of 60 fsw for six hours followed by rapid decompression consistently produces osteonecrosis in 10 of 13 animals. This dive profile is associated with increased utilization of platelets and fibrinogen indicating that thrombotic occlusion of the microvascular bed may lead to osteonecrosis.

Further studies have shown that depending on the dive profile similar hemostatic changes may be seen in man, and that these may not be

detectable by simply measuring concentrations of hemostatic factors in the blood stream since increased rates of production and changes in storage pools may compensate to maintain constant blood levels of coagulation factors.

Using a dive profile of 80 fsw for four hours in the swine followed by rapid decompression, a one and a half fold increase in platelet-fibrinogen destruction resulted. Prevention of this increased consumption occurred with combined treatment with platelet function inhibitors and anticoagulants. However, this therapy was not able to prevent primary pathologic changes since cessation of therapy promptly resulted in similar increases in platelet and fibrinogen destruction as were found in the untreated controls. After this dive profile fibrinogen survivals promptly normalized within one week while platelet survivals required four weeks suggesting vascular endothelial damage as a result of hyperbaric exposure which results in continued shortening of platelet survivals until re-endothelialization following injury is completed. Histologic studies in the animals confirmed loss of endothelium.

At least three important variables which adversely affect hemostasis after hyperbaric exposure are the depth of diving, the decompression profile used and the amount of tissue oxygenization. Decreased tissue oxygen tension during diving enhances the hemostatic abnormalities, as does uncontrolled descent from depth.

Whether monitoring dive profiles with hemostatic kinetic measurements may allow one to modify the dive profile to prevent thrombotic complications or alternatively may permit selection of appropriate antithrombotic therapy remains to be determined.

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TABLE I
HEMOSTATIC KINETICS POST-DIVING (HUMAN)

| ERVATIONS | DIVE PROFILE | | | | PLATELETS | | | | | FIBRINOGEN | | | | PLASMINOGEN | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Depth (Feet) | Bottom Time (min.) | Dives (#) | Week | Count (plat/ul) | Recovery (%) | Survival (days) | Turnover (plat/ul/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (mgm/ml/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (mgm/ml/day) | Times Normal |
| 20 | | (Normal | Values) | | 250,000 ± 5,000 | 65 ± 1 | 9.5 ±0.1 | 35,000 ± 2,000 | 1.0 | 2.95 ±0.20 | 5.1 ±0.1 | 0.57 ±0.02 | 1.0 | 105 ± 4 | 2.3 ±0.1 | 45 ± 2 | 1.0 |
| 4 | 100* | 60 | 1 | 0 | 213,000 ±15,000 | 64 ± 4 | 8.1 ±0.3 | 37,000 ± 4,000 | 1.1 ±0.1 | 2.46 ±0.04 | 4.0 ±0.1 | 0.59 ±0.03 | 1.1 ±0.1 | | | | |
| 4 | 100* | 60 | 3 | 0 | 240,000 ± 000 | 62 ± 4 | 6.1 ±0.2 | 58,000 ± 9,000 | 1.6 ±0.2 | 2.47 ±0.22 | 3.7 ±0.2 | 0.69 ±0.07 | 1.2 ±0.1 | | | | |
| 4 | 100 | 60 | 7 | 1 | 242,000 ±32,000 | 96 ±14 | 5.2 ±0.3 | 54,000 ±10,000 | 1.6 ±0.3 | 2.66 ±0.24 | 4.8 ±0.5 | 0.58 ±0.08 | 1.0 ±0.2 | | | | |
| 4 | 100 | 60 | 10 | 2 | 238,000 ±40,000 | 108 ± 9 | 4.4 ±0.2 | 55,000 ± 9,000 | 1.6 ±0.3 | 2.57 ±0.23 | 4.6 ±0.4 | 0.57 ±0.05 | 1.0 ±0.1 | | | | |
| 4 | 100 | 60 | 14 | 3 | 245,000 ±45,000 | 101 ± 9 | 5.5 ±0.7 | 48,000 ±11,000 | 1.4 ±0.3 | 2.76 ±0.21 | 4.7 ±0.2 | 0.54 ±0.05 | 1.0 ±0.1 | | | | |
| 5 | 150 | 10 | 1 | 0 | 203,000 | 64 | 6.7 | 48,000 | 1.4 | 2.19 | 4.3 | 0.51 | 1.0 | 67 | 2.2 | 30 | 0.7 |
| | 150 | 15 | 1 | | ±18,000 | ± 8 | ±0.6 | ± 9,000 | ±0.3 | ±0.19 | ±0.2 | ±0.05 | ±0.1 | ±16 | ±0.5 | ± 1 | |
| 4 | 200 | 60 | 1 | 0 | 249,000 ±50,000 | 72 ±12 | 7.0 ±0.8 | 49,000 ± 9,000 | 1.4 ±0.3 | 2.84 ±0.15 | 4.6 ±0.3 | 0.61 ±0.02 | 1.2 ±0.1 | 104 ±14 | 2.1 ±0.2 | 54 ±10 | 1.2 ±0.2 |
| 8 | 220 | 20 | 1 | 0 | 282,000 ±16,000 | 76 ± 4 | 6.4 ±0.4 | 53,000 ± 2,000 | 1.5 ±0.1 | 2.45 ±0.09 | 4.3 ±0.2 | 0.59 ±0.04 | 1.1 ±0.1 | 112 ±10 | 1.8 ±0.2 | 68 ±11 | 1.5 ±0.2 |
| 4 | | | | 1 | 314,000 ±38,000 | 82 ± 5 | 7.5 ±0.2 | 48,000 ± 5,000 | 1.4 ±0.1 | 2.46 ±0.34 | 4.8 ±0.4 | 0.52 ±0.07 | 1.0 ±0.2 | | | | |

TABLE I
HEMOSTATIC KINETICS POST-DIVING (HUMAN)

| SERVATIONS | DIVE PROFILE | | | | PLATELETS | | | | | FIBRINOGEN | | | | PLASMINOGEN | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Depth (Feet) | Bottom Time (min.) | Dives (#) | Week | Count (plat/ul) | Recovery (%) | Survival (days) | Turnover (plat/ul/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (mgm/ml/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (ug/ml/day) | Times Normal |
| 3 | 300** | 30 | 1 | 0 | 283,000 | 72 | 8.5 | 42,000 | 1.2 | 2.89 | 4.1 | 0.78 | 1.3 | 140 | 1.6 | 90 | 2.0 |
| | | | | | ±58,000 | ± 5 | ±0.2 | ± 8,000 | ±0.2 | ±0.44 | ±0.1 | ±0.16 | ±0.2 | ± 4 | ±0.1 | ± 1 | ±0.0 |
| 2 | 400** | 60 | 1 | 0 | 306,000 | 73 | 5.9 | 70,000 | 2.0 | 3.57 | 5.4 | 0.70 | 1.3 | 147 | 1.6 | 142 | 3.2 |
| | | | | | ± 3,000 | ±15 | ±0.9 | ± 0 | ±0.0 | ±0.13 | ±0.6 | ±0.24 | ±0.5 | ± 6 | ±0.4 | ±11 | ±0.3 |
| 1 | 500** | 60 | 1 | 0 | 263,000 | 56 | 5.2 | 73,000 | 2.1 | 2.44 | 4.9 | 0.50 | 0.9 | | | | |
| 2 | 700** | 60 | 1 | 0 | 216,000 | 61 | 2.9 | 107,000 | 3.1 | 2.80 | 3.3 | 0.86 | 1.6 | | | | |
| | | | | | ±62,000 | ± 1 | ±0.4 | ±15,000 | ±0.5 | ±0.11 | ±0.3 | ±0.11 | ±0.2 | | | | |
| | | | | 1 | 235,000 | 53 | 8.6 | 46,000 | 1.4 | 2.91 | 4.4 | 0.68 | 1.3 | | | | |
| | | | | | ±63,000 | ± 4 | ±0.1 | ± 9,000 | ±0.3 | ±0.06 | ±0.5 | ±0.09 | ±0.2 | | | | |

* Royal Navy Decompression Schedule

** Praeger gas

TABLE II

PLATELET AND FIBRINOGEN CONCENTRATIONS (HUMAN)

| OBSERVATIONS | DIVE PROFILE | | PLATELET COUNT % of Baseline day post-dive | | | FIBRINOGEN CONCENTRATION % of Baseline day post-dive | | |
|---|---|---|---|---|---|---|---|---|
| | DEPTH (feet) | BOTTOM TIME (minutes) | 1 | 2 | 3 | 1 | 2 | 3 |
| 4 | 100 | 60 | 93.0 ±4.4 | 93.0 ±2.6 | 100 ±3.4 | 104.8 ±7.7 | 97.3 ±3.6 | 98.8 ±5.4 |
| 5 | 150<br>150 | 10<br>15 | 102.6 ±3.4 | 96.2 ±4.5 | 99.4 ±4.0 | 103.2 ±5.1 | 107.0 ±5.9 | 110 ±3.5 |
| 4 | 200 | 60 | 102.3 ±3.4 | 98.3 ±2.6 | 101.8 ±1.9 | 105.7 ±2.4 | 99.5 ±1.8 | 103.8 ±4.1 |
| 4 | 158 | 60 | 99.8 ±4.3 | 102.7 ±3.6 | 98.0 ±4.1 | 98.3 ±2.9 | 101.3 ±6.6 | 92.3 ±7.0 |
| 12 | 220 | 20 | 96.5 ±3.0 | 98.1 ±2.9 | 103.3 ±2.9 | 96.2 ±3.2 | 95.5 ±7.3 | 97.8 ±5.5 |
| 3 | 300 | 30 | 98.0 ±3.6 | 93.4 ±1.9 | 104.0 ±3.6 | 100.1 ±0.9 | 101.0 ±2.0 | 101.3 ±1.2 |
| 2 | 400 | 60 | 107.0 ±13.1 | 113.5 ±16.6 | 108.5 ±18.6 | 102.5 ±5.4 | 99.7 ±0.4 | 105.4 ±1.7 |
| 1 | 500 | 60 | 88.6 | 95.4 | - | 96.4 | 96.4 | 89.6 |
| 2 | 700 | 150 | 85 ±13 | 83 ±8 | 110 ±2 | 125 ±8 | 97 ±6 | 104 ±6 |

TABLE III

OXYGEN CONCENTRATION (HUMAN)

| OBSERVATIONS | DIVE PROFILE | | | | PLATELETS | | | | | FIBRINOGEN | | | | PLASMINOGEN | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Depth (Feet) | Bottom Time (min.) | Dives (#) | Week | Count (plat/ul) | Recovery (%) | Survival (days) | Turnover (plat/ul/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (mgm/ml/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (ug/ml/day) | Times Normal |
| 4 | 158* | 60 | 1 | 0 | 242,000 | 57 | 6.6 | 61,000 | 1.7 | 2.83 | 3.8 | 0.76 | 1.4 | 75 | 1.9 | 43 | 0.9 |
| | | | | | ±23,000 | ± 5 | ±1.0 | ± 5,000 | ±0.2 | ±0.20 | ±0.2 | ±0.07 | ±0.1 | ± 4 | ±0.1 | ± 4 | ±0.1 |
| 4 | 200 | 60 | 1 | 0 | 249,000 | 72 | 7.0 | 49,000 | 1.4 | 2.84 | 4.6 | 0.61 | 1.2 | 104 | 2.1 | 54 | 1.2 |
| | | | | | ±50,000 | ±12 | ±0.8 | ± 9,000 | ±0.3 | ±0.15 | ±0.3 | ±0.02 | ±0.1 | ±14 | ±0.2 | ±10 | ±0.2 |

* 96% nitrogen, 4% oxygen gas mixture

TABLE IV

TREATMENT WITH PLATELET FUNCTION INHIBITORS (HUMAN)

| OBSERVATIONS | DIVE PROFILE | | | | PLATELETS | | | | | FIBRINOGEN | | | | PLASMINOGEN | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Depth (Feet) | Bottom Time (min.) | Dives (#) | Week | Count (plat/ul) | Recovery (%) | Survival (days) | Turnover (plat/ul/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (mgm/ml/day) | Times Normal | Conc. (mgm/ml) | Survival (days) | Turnover (ug/ml/day) | Times Normal |
| 4 | 220 | 20 | 2 | 0 | 235,000 | 71 | 5.2 | 58,000 | 1.7 | 2.46 | 4.4 | 0.56 | 1.0 | | | | |
| | | | | | ±25,000 | ± 1 | ±0.5 | ± 2,000 | ±0.3 | ±0.16 | ±0.1 | ±0.04 | ±0.1 | | | | |
| 4* | 220 | 20 | 2 | 0 | 226,000 | 62 | 7.3 | 46,000 | 1.3 | 2.20 | 4.5 | 0.49 | 0.9 | | | | |
| | | | | | ±21,000 | ± 3 | ±0.4 | ± 6,000 | ±0.2 | ±0.24 | ±0.2 | ±0.06 | ±0.1 | | | | |

* All divers were taking 225 mg dypridamole and 960 mg ASA per day in three divided doses

TABLE V

HEMOSTATIC KINETICS (SWINE)

| | | Platelets | | | | | Fibrinogen | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Post Dive (Week)* | Observations (#) | Count plat/µl | Recovery % | Survival days | Turnover plat/µl/day | x N | Concentration mg/ml | Survival days | Turnover mg/ml/day | x N |
| Baseline (-1) | 19 | 329,000 ±25,000 | 66 ±4 | 5.2 ±0.1 | 88,000 ± 9,000 | 1.0 | 4.37 ±0.27 | 3.6 ±0.1 | 1.23 ±0.08 | 1.0 |
| 0 | 7 | 291,000 ±50,000 | 93 ±5 | 2.3 ±0.3 | 139,000 ±21,000 | 1.6 ±0.2 | 6.28 ±0.52 | 2.1 ±0.3 | 3.13 ±0.27 | 2.5 ±0.2 |
| 1 | 5 | 383,000 ±46,000 | 85 ±10 | 3.5 ±0.4 | 128,000 ±15,000 | 1.5 ±0.2 | 5.35 ±0.46 | 3.8 ±0.4 | 1.49 ±0.25 | 1.2 ±0.2 |
| 2 | 5 | 328,000 ±45,000 | 79 ±10 | 4.1 ±0.2 | 98,000 ±17,000 | 1.1 ±0.2 | 5.42 ±0.23 | 3.7 ±0.3 | 1.52 ±0.18 | 1.2 ±0.2 |
| 3 | 3 | 281,000 ±83,000 | 95 ±5 | 4.4 0.2 | 65,000 ±20,000 | 0.7 ±0.3 | 4.81 ±0.59 | 4.0 ±0.1 | 1.20 ±0.12 | 1.0 ±0.1 |
| 4 | 3 | 295,000 ±71,000 | 69 ±10 | 4.8 ±0.3 | 79,000 ±11,000 | 0.9 ±0.2 | 5.22 ±0.80 | 4.3 ±0.6 | 1.32 ±0.41 | 1.1 ±0.4 |

* Animals had a single dive of 80 fsw for 4 hours with decompression at 30 fpm.

TABLE VI

OXYGEN CONCENTRATION (SWINE)

| Post Dive (Week)* | Observations | Treatment | PLATELETS | | | | | FIBRINOGEN | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Count plat/µl | Recovery % | Survival days | Turnover plat/µl/day | x N | Concentration mg/ml | Survival days | Turnover mg/ml/day | x N |
| Baseline (-1) | 9 | None | 329,000 ±25,000 | 66 ±4 | 5.2 ±0.1 | 88,000 ±9,000 | 1.0 | 4.37 ±0.2 | 3.6 ±0.1 | 1.23 ±0.08 | 1.0 |
| - | 2 | 100% Oxygen on surface | 361,000 ± 0,000 | >100.0 | 4.9 ±0.2 | 73,000 ±3,000 | 0.9 ±0.1 | 6.56 ±2.08 | 3.7 ±0.1 | 1.78 ±0.51 | 1.5 ±0.5 |
| 0 | 7 | Normoxic | 290,000 ±29,000 | 101.5 ±6.0 | 2.6 ±0.2 | 119,000 ±13,000 | 1.4 ±0.2 | 6.67 ±0.65 | 2.1 ±0.2 | 3.50 ±0.64 | 2.9 ±0.5 |
| 1 | 2 | None | 373,000 ±69,000 | 84.6 ±7.8 | 2.3 ±0.3 | 180,000 ±22,000 | 2.1 ±0.3 | 5.77 ±0.12 | 3.6 ±0.4 | 1.61 ±0.20 | 1.3 ±0.2 |
| 2 | 2 | None | 382,000 ±116,000 | 81.8 ±0.2 | 2.6 ±0.2 | 163,000 ±41,000 | 1.9 ±0.6 | 5.01 ±0.69 | 3.9 ±0.6 | 1.33 ±0.37 | 1.1 ±0.4 |
| 3 | 3 | None | 335,000 ±41,000 | 87.9 ±10.4 | 3.9 ±0.8 | 131,000 ±22,000 | 1.5 ±0.3 | 6.23 ±0.80 | 3.7 ±0.4 | 1.92 ±0.44 | 1.6 ±0.4 |
| 4 | 5 | None | 328,000 ±32,000 | 84.4 ±7.2 | 3.7 ±0.6 | 106,000 ±11,000 | 1.2 ±0.1 | 6.04 ±0.77 | 3.5 ±0.5 | 1.80 ±0.28 | 1.5 ±0.3 |
| 5 | 1 | None | 217,000 | 72.1 | 2.4 | 113,000 | 1.3 | 7.27 | 5.4 | 1.35 | 1.1 |
| 6 | 1 | None | 365,000 | 69.7 | 5.0 | 95,000 | 1.1 | 7.11 | 4.7 | 1.52 | 1.2 |
| 7 | 1 | None | 202,000 | 33.9 | 3.3 | 161,000 | 1.8 | 9.81 | 3.8 | 2.55 | 2.1 |
| 13 | 2 | None | 259,000 ±90,000 | 79.4 ±0.5 | 3.7 ±0.5 | 78,000 ±32,000 | 0.9 ±0.5 | 4.87 ±0.29 | 4.2 ±0.1 | 1.56 ±0.49 | 1.3 ±0.5 |

* Animals had a single dive of 80 fsw for 4 hours with decompression at 30 fpm

TABLE VII

ANTITHROMBOTIC TREATMENT (SWINE)

| | | | Platelets | | | | | Fibrinogen | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Post-Dive (Week)* | Observations | Treatment | Count plat/µl | Recovery % | Survival days | Turnover plat/µl/day | x N | Concentration mg/ml | Survival days | Turnover mg/ml/day | x N |
| Baseline (-1) | 19 | None | 329,000 ±25,000 | 66 ±4 | 5.2 ±0.1 | 88,000 ±9,000 | 1.0 | 4.37 ±0.2 | 3.6 ±0.1 | 1.23 ±0.08 | 1.0 |
| 0 | 6 | Platelet function inhibitor | 276,000 ±42,000 | 83 ±11 | 2.6 ±0.1 | 150,000 ±42,000 | 1.7 ±0.5 | 6.11 ±0.53 | 1.9 ±0.2 | 3.41 ±0.49 | 2.8 |
| 1 | 3 | Platelet function inhibitor | 424,000 ±59,000 | 78.6 ±12.7 | 5.0 ±0.3 | 100,000 ±13,000 | 1.2 ±0.2 | 6.70 ±0.23 | 3.9 ±0.1 | 1.71 ±0.09 | 1.4 |
| 1 | 1 | None | 263,000 | 101.3 | 3.2 | 83,000 | 0.9 | 7.99 | 2.9 | 2.70 | 2.2 |
| 2 | 4 | None | 271,000 ±67,000 | 73.3 ±5.1 | 3.4 ±0.2 | 97,000 ±24,000 | 1.1 ±0.4 | 6.22 ±1.48 | 3.9 ±0.1 | 1.55 ±0.40 | 1.3 ±0.4 |
| 3 | 3 | None | 403,000 ±103,000 | 85.1 ±5.6 | 4.9 ±0.3 | 93,000 ±31,000 | 1.1 ±0.4 | 4.84 ±0.32 | 3.9 ±0.1 | 1.24 ±0.07 | 1.0 ±0.1 |
| 4 | 1 | None | 221,000 | 59.8 | 3.5 | 96,000 | 1.1 | 4.03 | 4.1 | 0.97 | 0.8 |
| 0 | 4 | Anti-coagulant | 298,000 ±35,000 | 85.1 ±4.8 | 3.1 ±0.1 | 102,000 ±10,000 | 1.2 ±0.2 | 6.78 ±0.55 | 2.2 ±0.1 | 3.14 ±0.31 | 2.6 ±0.3 |
| 1 | 3 | Anti-coagulant | 353,000 ±45,000 | 46.3 ±4.5 | 4.5 ±0.2 | 161,000 ±20,000 | 1.8 ±0.3 | 8.02 ±0.25 | 4.7 ±0.6 | 1.74 ±0.18 | 1.4 ±0.2 |
| 0 | 3 | Anti-coagulant + Platelet function inhibitor | 343,000 ±19,000 | 59.2 ±17.5 | 4.7 ±0.4 | 88,000 ±1,000 | 1.0 ±0.0 | 6.63 ±1.48 | 3.5 ±0.5 | 2.10 ±0.77 | 1.7 ±0.7 |

* Animals had a single dive of 80 fsw with decompression at 30 fpm

TABLE VIII

| Animal | Aortic Endothelial Cell Loss (%) |
|---|---|
| untreated | |
| 1 | 5 |
| 2 | 4 |
| 3 | 10 |
| 4 | 25 |
| 5 | 21 |
| 6 | 7 |
| 7 | 4 |
| 8 | 15 |
| mean | 11 $\pm$ 6 |
| treated | |
| 9 | 11 |
| 10 | 6 |
| 11 | 12 |
| 12 | 6 |
| 13 | 3 |
| 14 | 9 |
| 15 | 15 |
| mean | 9 $\pm$ 4 |

## Figures

Figure 1

Platelet and fibrinogen survival studies in four divers after 1, 3, 7, 10 and 14 daily dives of 100 fsw for 60 minutes using British Royal Navy Decompression Tables. Results are expressed as the average ± 1 S.E.

Figure 2

Platelet (Part A) and plasminogen (Part B) survivals compared to fibrinogen survivals in individual divers following the dive profiles outlined in Table I. There is only a poor correlation between these measurements. Normal survivals ± 1 standard error are shown in the hatched areas.

Figure 3

Comparison of platelet (Part A) and plasminogen (Part B) turnovers with fibrinogen turnovers in individual divers following dive profiles outlined in Table I. Platelet turnover is either comparable to fibrinogen or disproportionally shortened while plasminogen and fibrinogen turnovers are basically proportional. Normal turnovers ± I S.E. are shown in the hatched area.

Figure 4

Relationship between depth of diving and platelet survival for dives of 60 minute duration plotted as the average ± I S.E.

Figure 5

Changes in platelet count, recovery and survival in swine followed serially after a single dive of 80 fsw for 4 hours with decompression at 30 fsw/minute. Results are expressed as average values ± I S.E.

Figure 6

Changes in fibrinogen concentration and survival in swine followed serially after a single dive of 80 fsw for 4 hours with decompression at 30 fsw/minute. Results are expressed as average values ± 1 S.E.

Figure 7

Representative platelet survival in an animal following repetitive exposure to a dive profile of 60 fsw for 6 hours 3 times a week for 13 weeks with decompression at 30 fsw/minute. Open circles (○) show platelet survival without antithrombotic treatment while (▲) indicated studies done during administration of coumarin plus 10 mg persantin/kilo/day and 600 mg aspirin in three divided doses. Platelet survival progressively shortened with repeated exposure but normalizes with administration of antithrombotic therapy. Seven weeks after cessation of diving (week 20) platelet survival had returned towards normal. The normal platelet survival in swine ± 1 S.D. is shown by the hatched area.

Figure 8

Serial platelet (Part A) and fibrinogen (Part B) survivals in swine following a single normoxic dive of 80 fsw for 4 hours with decompression at 30 fsw/minute. Platelet survivals are still shortened from normal values shown in the hatched areas at 4 weeks while fibrinogen survivals are normal at one week. Results are shown as average values ± 1 S.E.

Figure 9

Following a dive of 80 fsw for 4 hours platelet (Part A) and fibrinogen (Part B) survival in 7 swine progressively returned to normal (hatched area ± 1 S.D.) after 4 weeks for platelets in swine and after one week for fibrinogen as shown by the closed circles (●), while treatment with platelet function inhibitors in 7 animals shown as the

open circles (◯) or anticoagulant in 4 animals shown as the closed triangles (▲) show no effect in the immediate post-dive period on either platelet or fibrinogen survivals but they are normal by the first post-dive week. Administration of both anticoagulants plus platelet function inhibitors normalized platelet and fibrinogen survivals in the immediate post-dive period as shown by the open squares (□). Results are shown as average values ± 1 S.E.

Figure 10

Silver stain of aortic endothelial cell surface demonstrating loss.

Figure 1

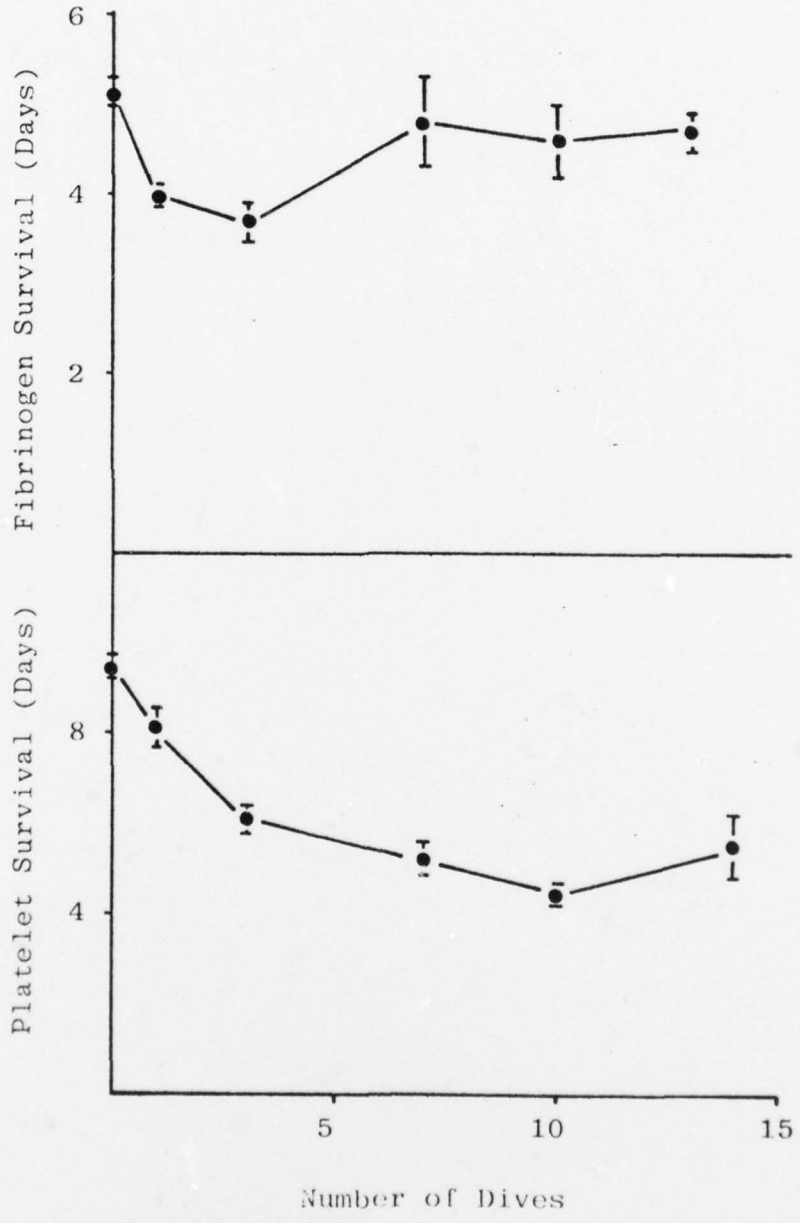

6
4
2
Fibrinogen Survival (Days)
8
4
Platelet Survival (Days)
5
10
15
Number of Dives

Figure 2

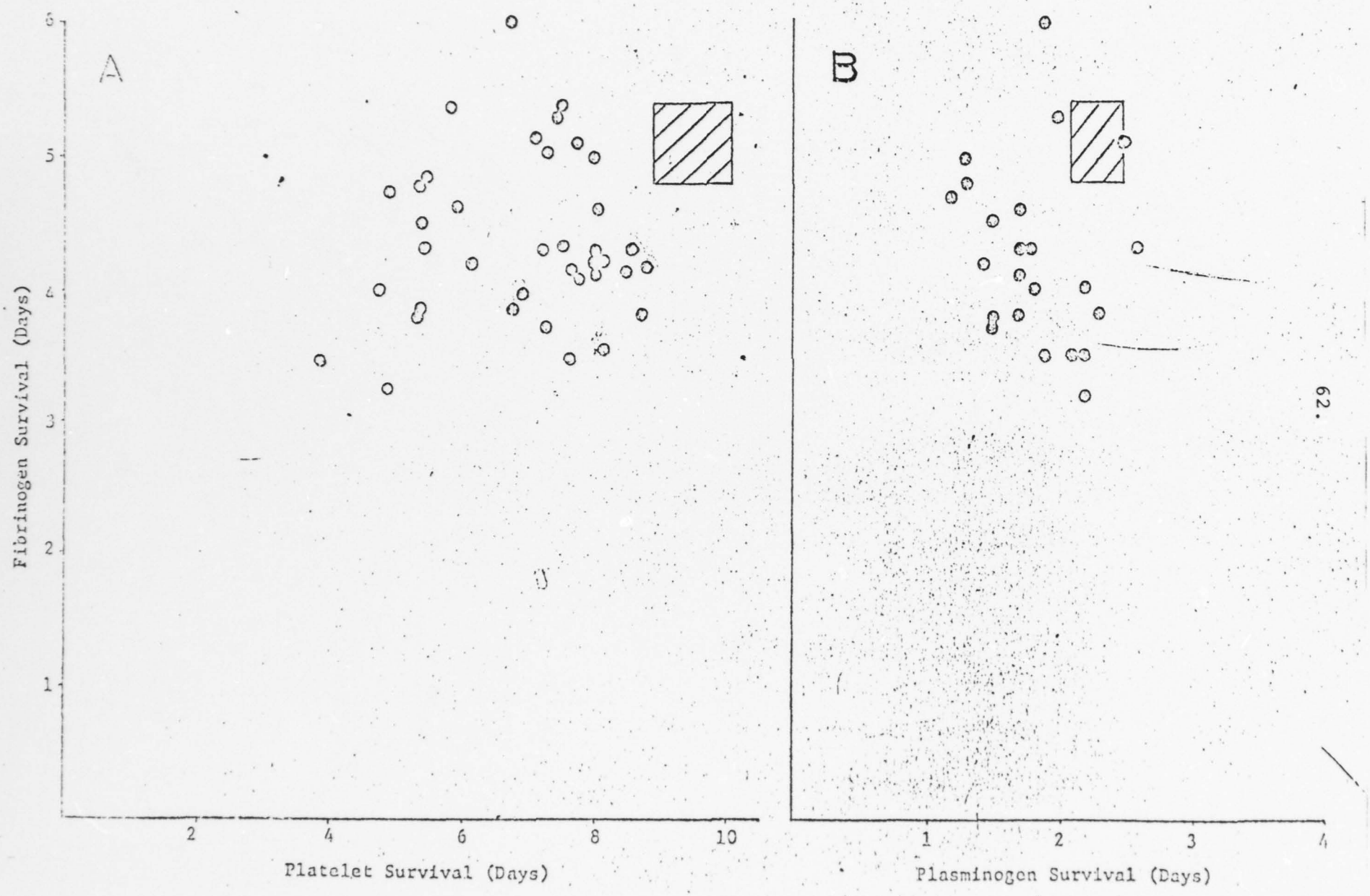
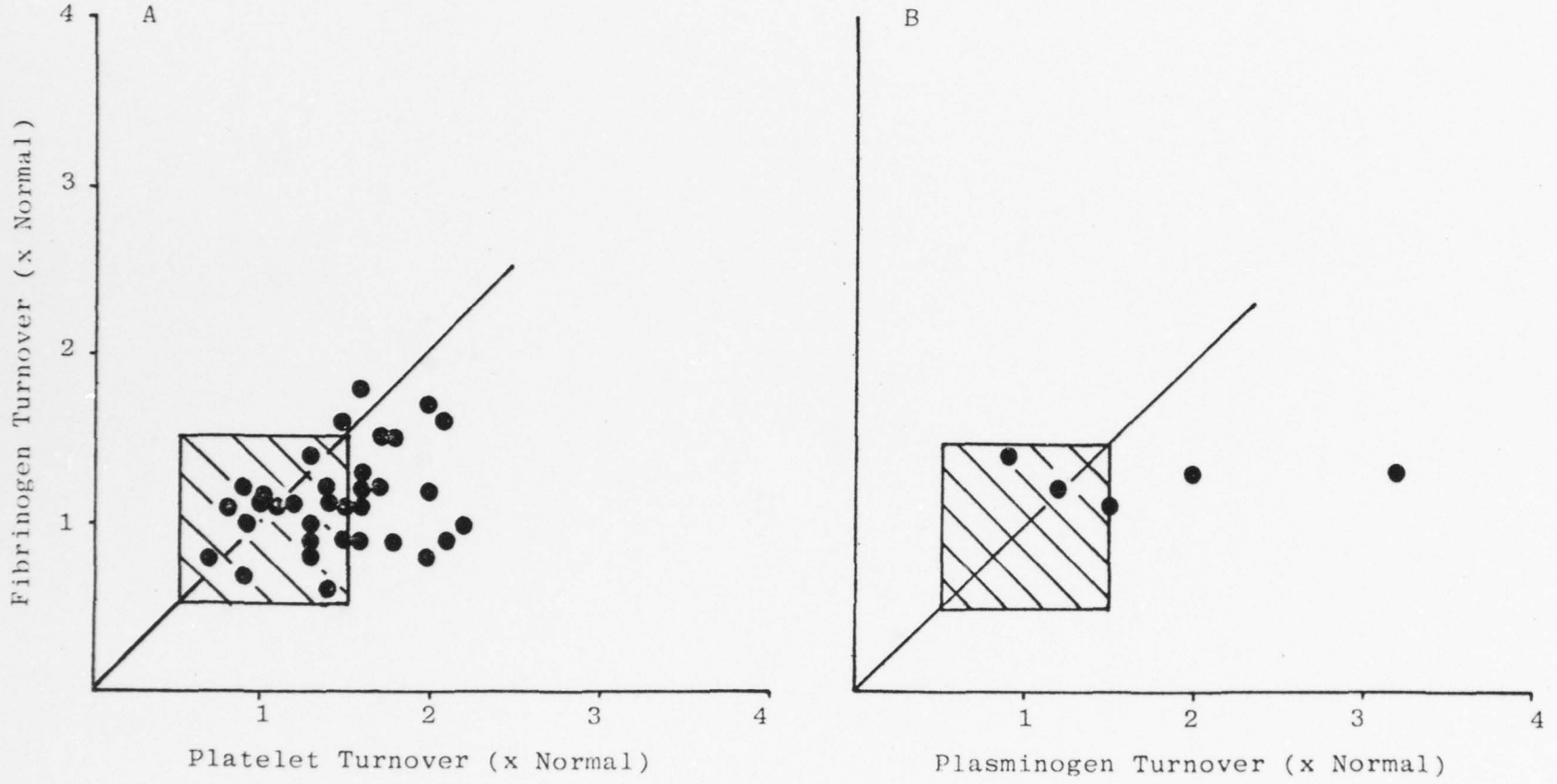

A
B
Fibrinogen Survival (Days)
Platelet Survival (Days)
Plasminogen Survival (Days)
1
2
3
4
5
6
2
4
6
8
10
1
2
3
4

Figure 3


A
B
Fibrinogen Turnover (x Normal)
Platelet Turnover (x Normal)
Plasminogen Turnover (x Normal)
1
2
3
4

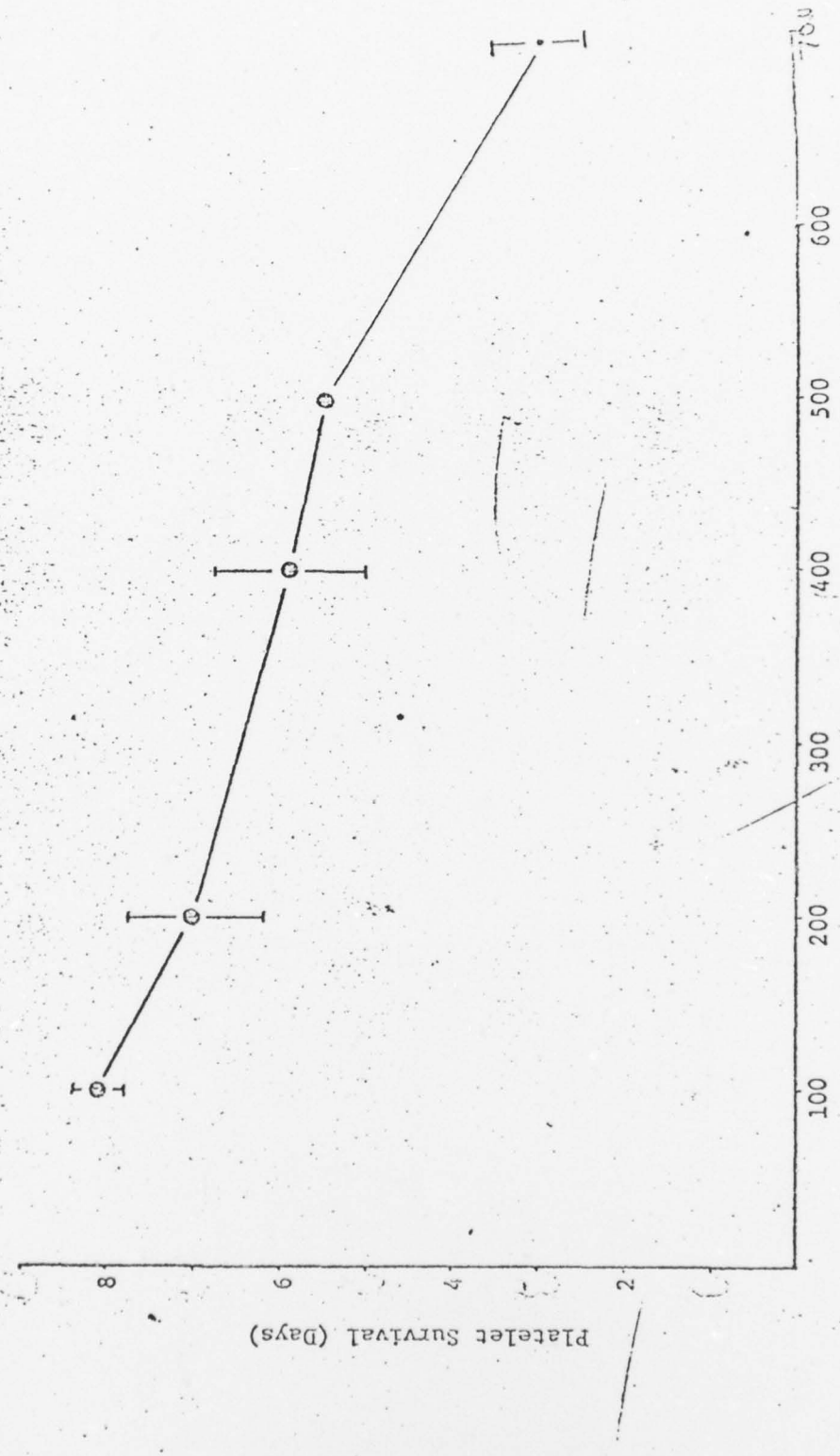
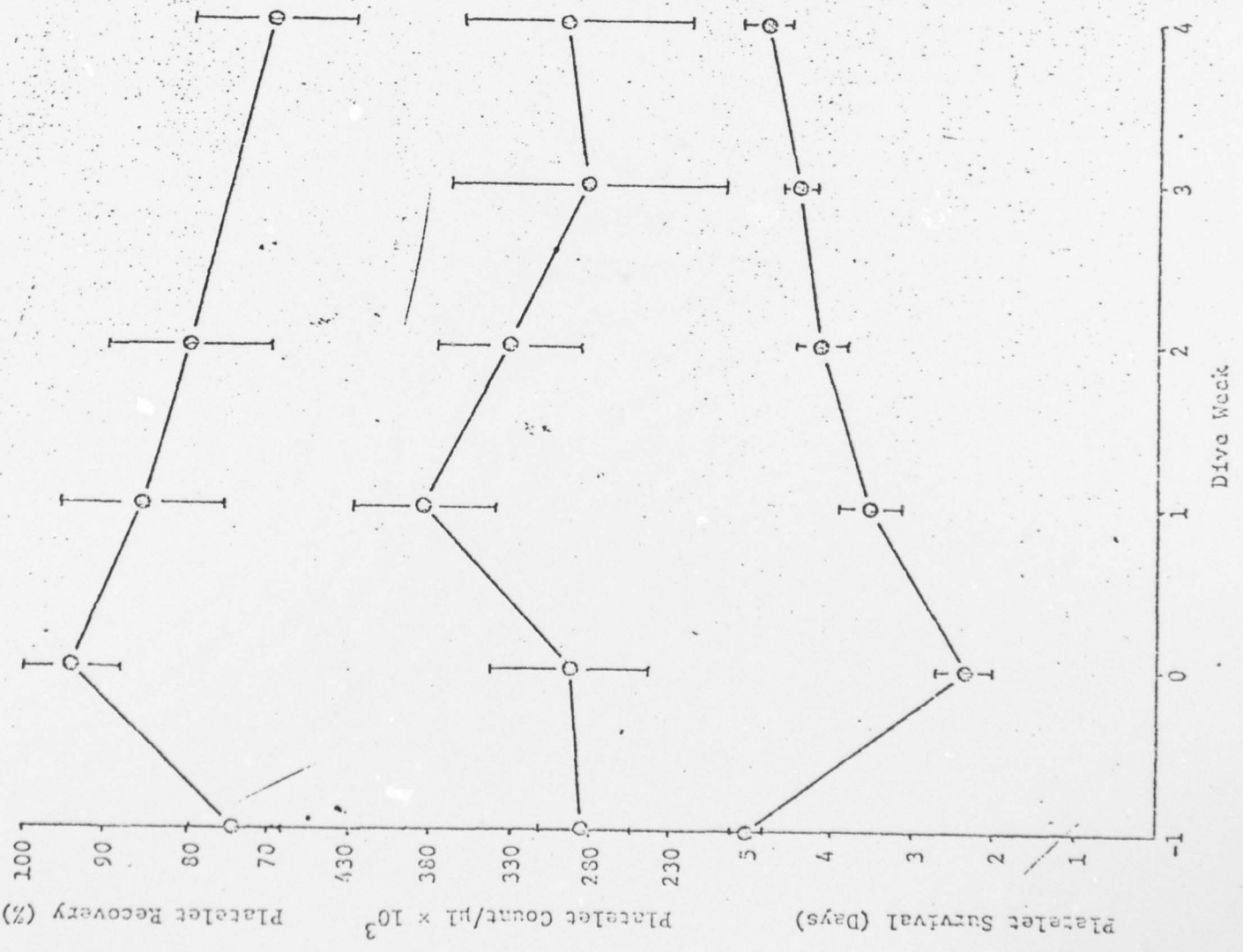

Platelet Survival (Days)
2
4
6
8
Depth (Feet)
100
200
300
400
500
600
700

Figure 4

Figure 5


Platelet Survival (Days)
Platelet Count/μl x 10³
Platelet Recovery (%)
Dive Week
-1
0
1
2
3
4
1
2
3
4
5
230
280
330
380
430
70
80
90
100

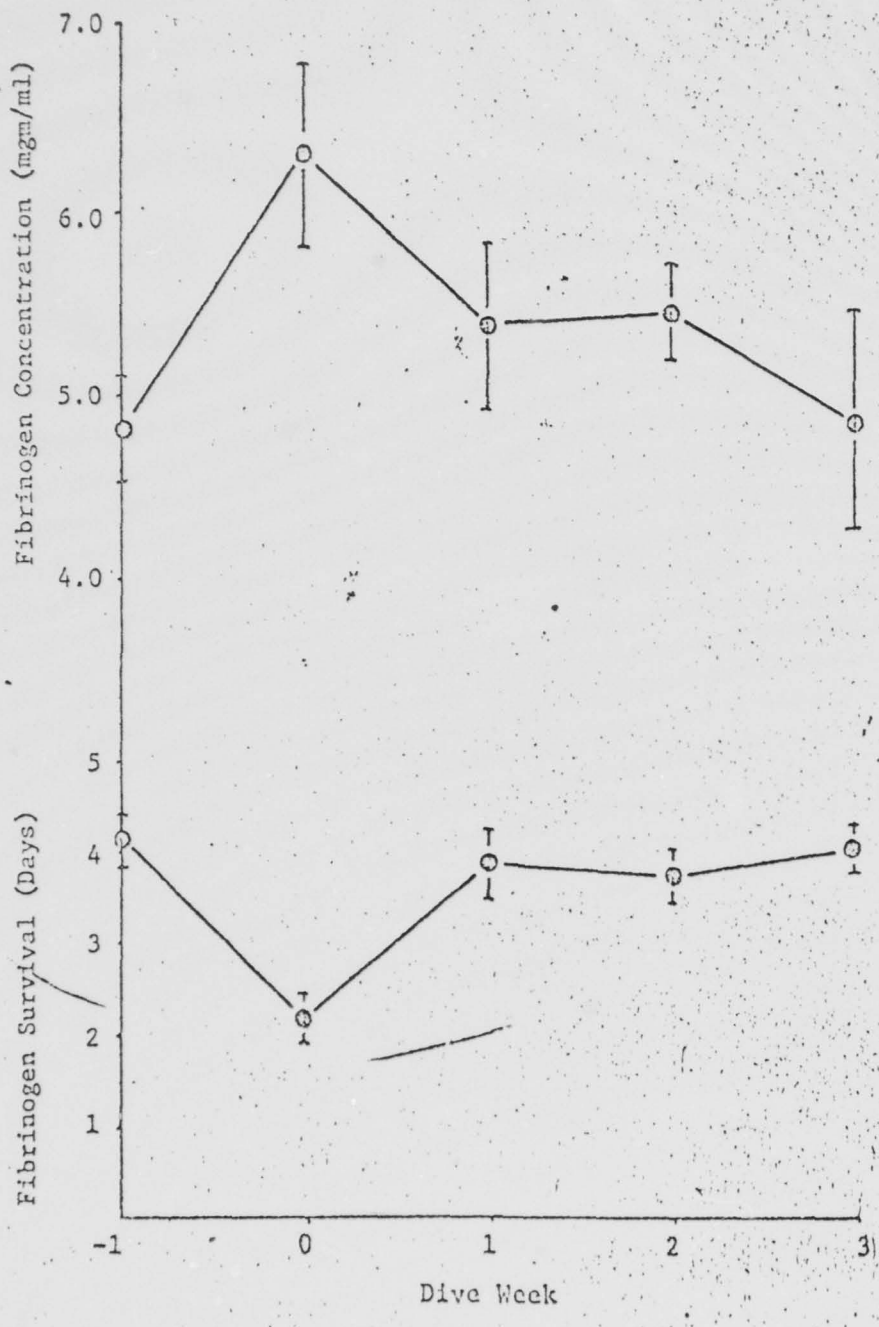
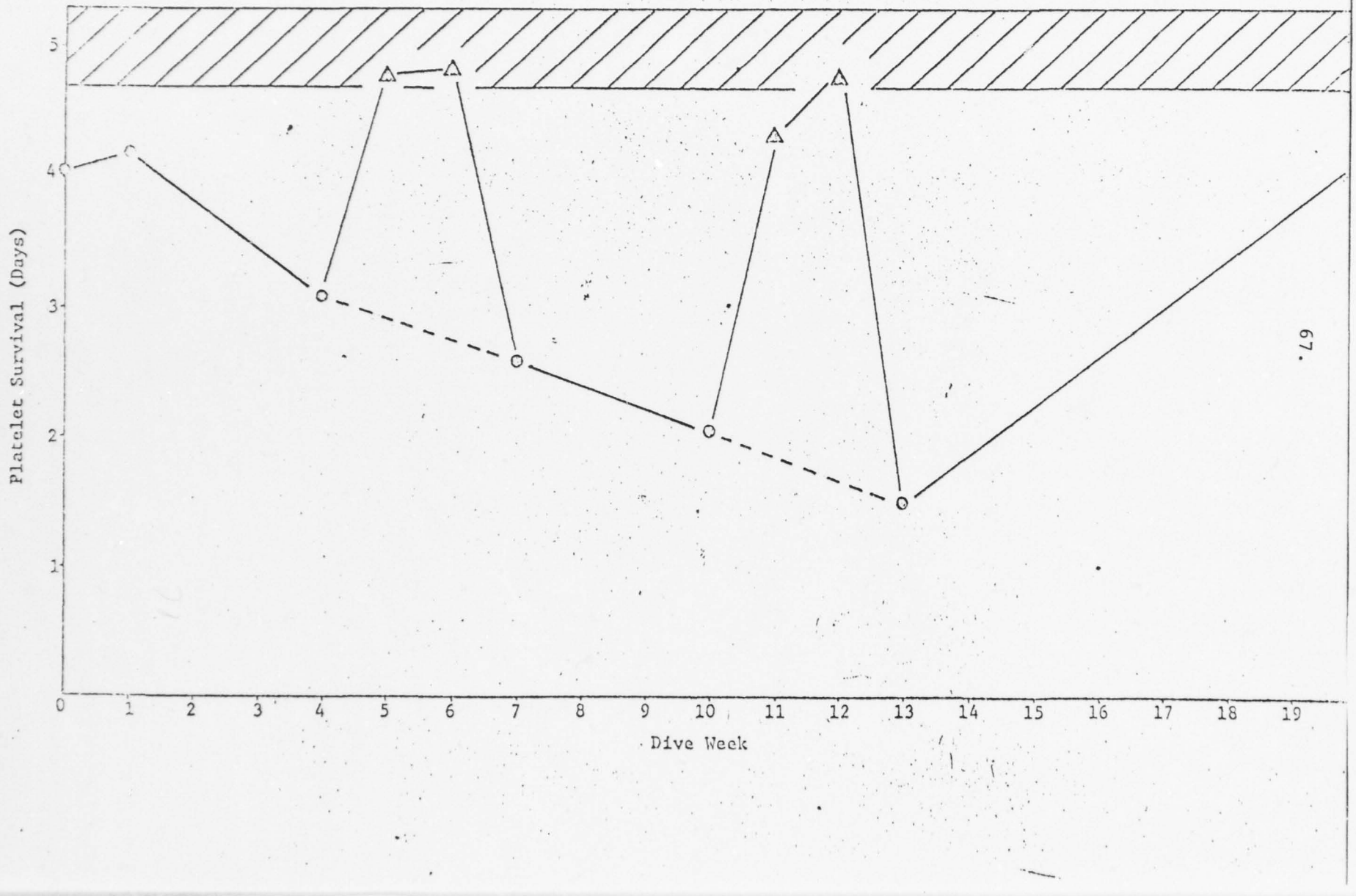

7.0
6.0
5.0
4.0
Fibrinogen Concentration (mgm/ml)
5
4
3
2
1
Fibrinogen Survival (Days)
-1
0
1
2
3
Dive Week

Figure 6

Figure 7


Platelet Survival (Days)
0
1
2
3
4
5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Dive Week

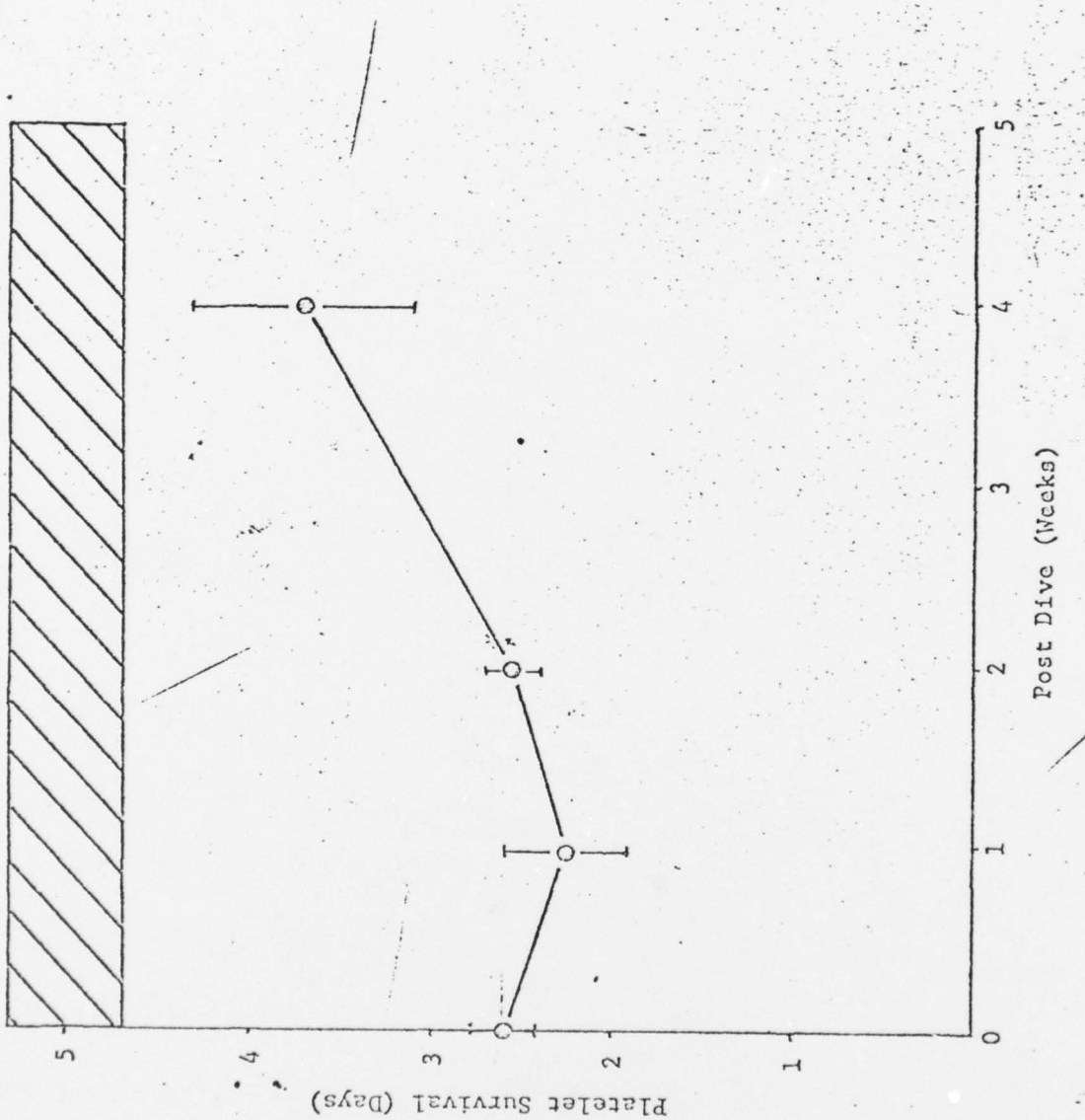



Figure 8A

Figure 8B

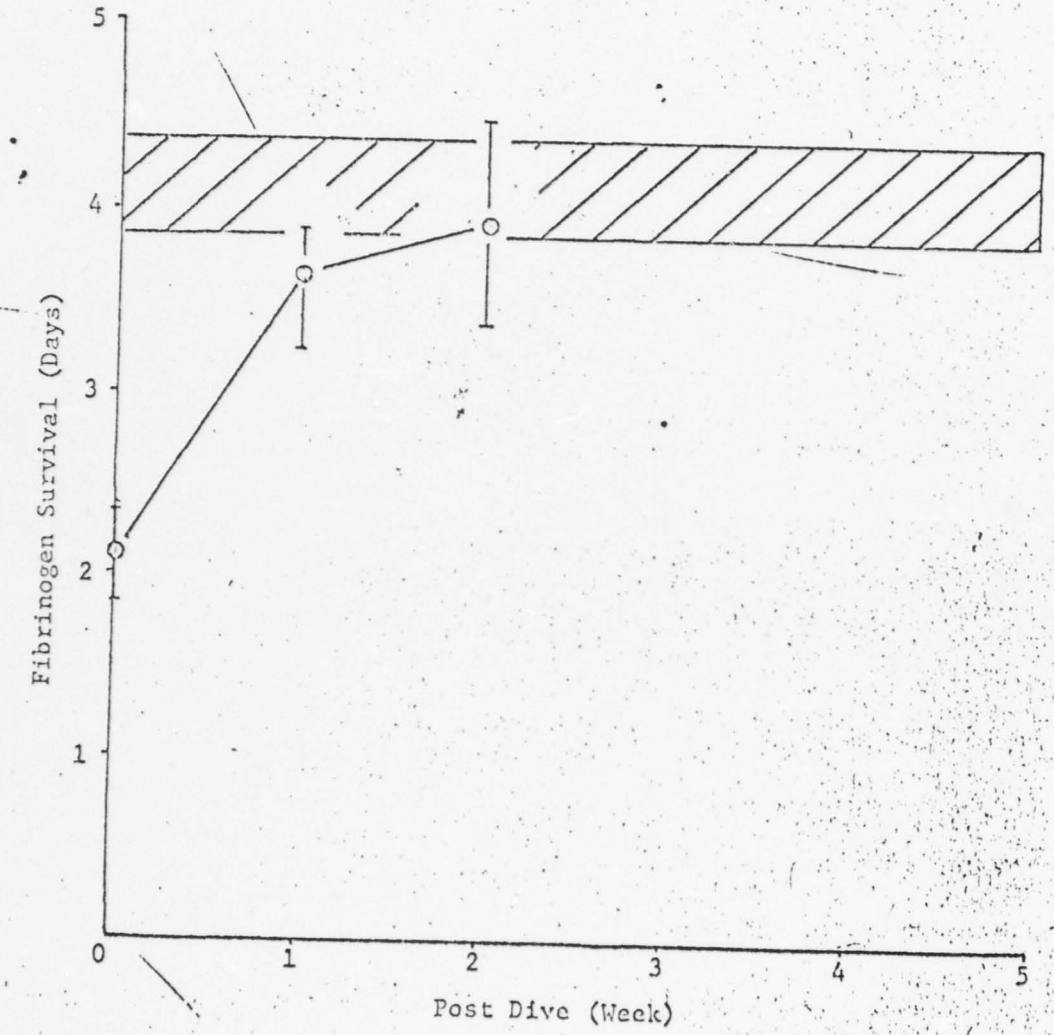


Figure 9A

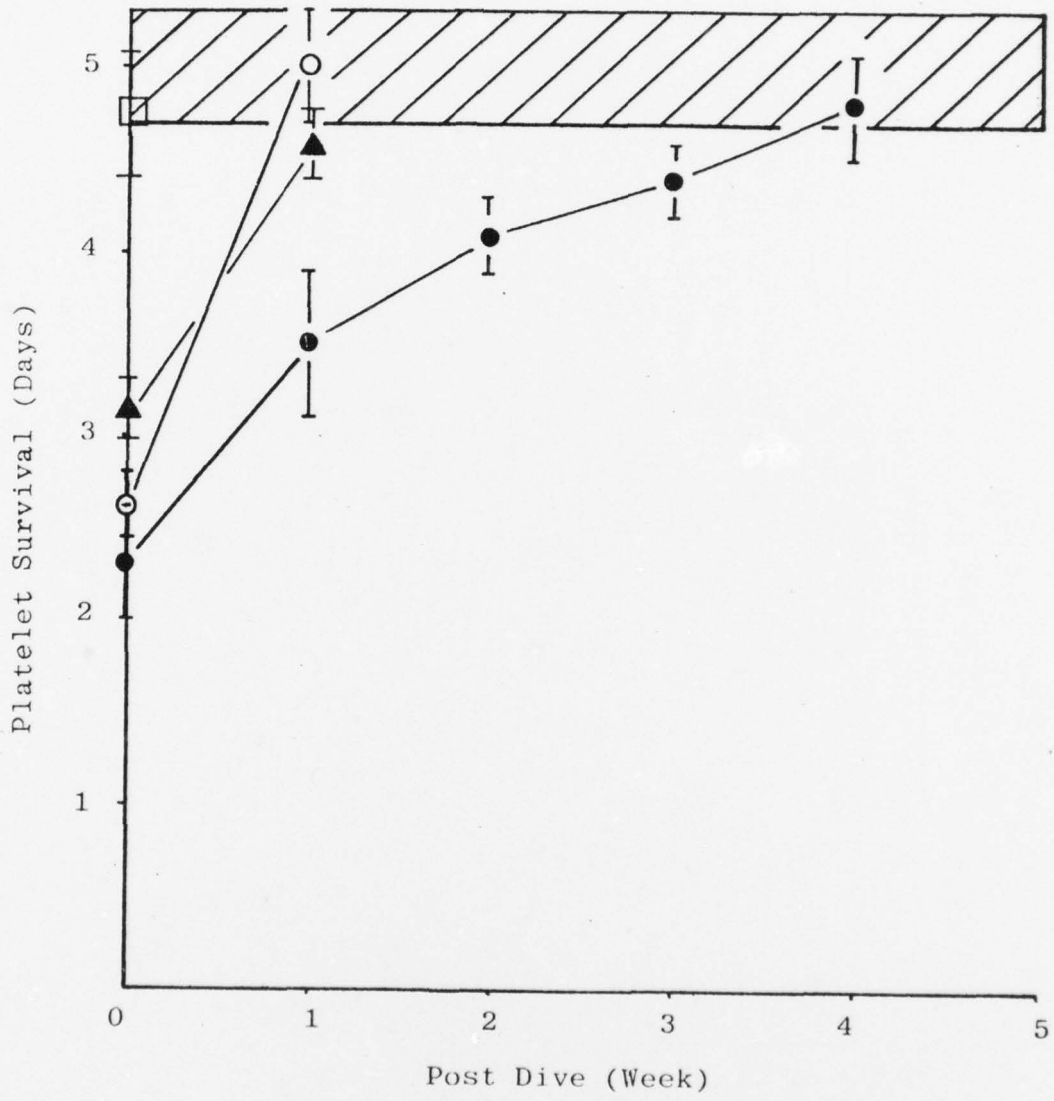


Figure 9B

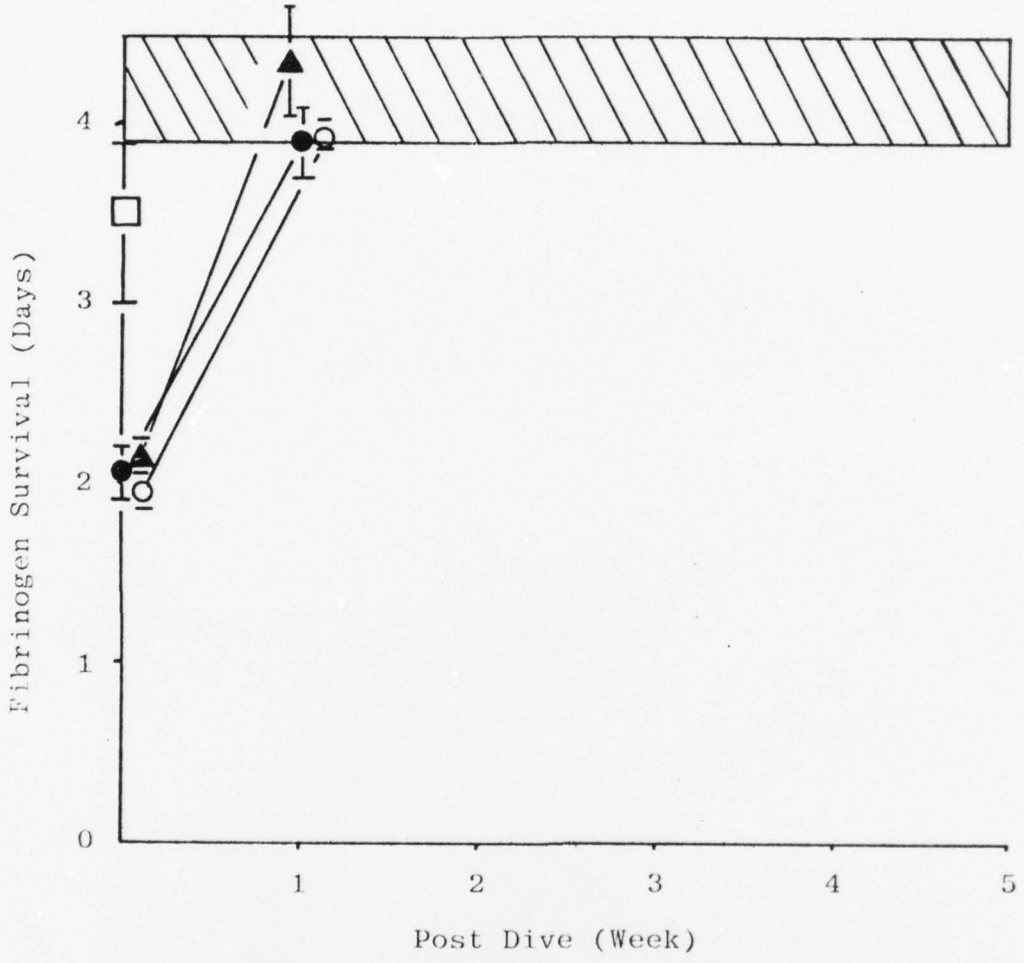
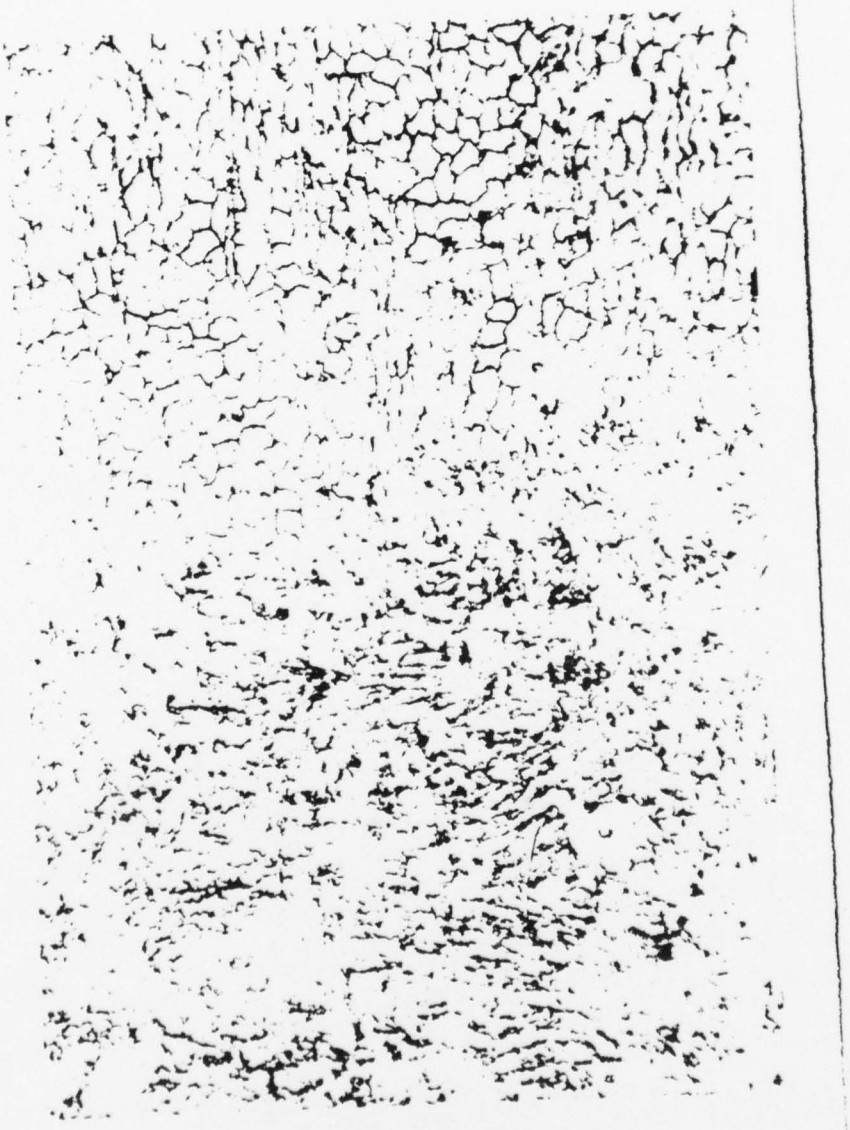


Figure 10



III Histopathology

One of the most significant aspects of our studies was our histologic investigation of both old and recent decompression injury. The significance stems from the confirmation of hypotheses regarding the etiology of osteonecrosis, and perhaps other pathology, associated with decompression.

The first confirmation was that the injury occurred with the first decompression insult. This hypothesis was presented when this study began, but conclusive evidence was obtained that indeed, histopathologic signs were observable within hours of the first decompression insult.

The second significant corroboration of a hypothesis was the definite documentation of endothelial injury, myointimal proliferation and hyaline clot formation in early osteonecrosis. We had hypothesized that endothelial damage was directly responsible for increased platelet and fibrinogen utilization and that there was undoubtedly some relationship to dysbaric osteoncreosis.

The following report of progress details some of the evidence which substantiates these hypotheses.

A. Specimen Preparation

Soft tissues obtained for routine histologic study were immediately fixed in buffered formalin, and after fixation were paraffin-embedded, sectioned by microtome, stained with hematoxylin and eosin, and mounted for basic histopathologic study. Femurs

and humeri were removed from each animal intact, and after being halved on the longitudinal axis, were fixed in buffered formalin, decalcified to radiolucency and finally double-embedded in paraffin and nitrocellulose. Ten micra sections of the whole head and proximal shaft of each bone were obtained before the large specimen block was quartered, allowing 5-6 micra sections to be cut.

Areas of change seen grossly or radiographically were selectively chosen for electron microscopy. Tissues from these areas were fixed in glutaraldehyde, stained with lead hydrozide, embedded in Epon 812, and sectioned on a Porter-Blum MT-1 microtome for ultramicroscopic study.

B. General Histopathologic Findings

The most prominent finding was extensive hyaline thrombosis (Fig. 1, 3) of small vessels in the Haversian canals of cortical bone. This was associated with cortical bone necrosis. Subsequent reparative attempt of bone led to resorption of necrotic bone, deposition of new lamellar bone (Fig. 2, 4) on inner surfaces of widened Haversian canals, and subendosteal new bone formation. The osteons appeared disorganized and separated by necrotic interstitial bone plates.

Figure 1 A hyaline clot (arrow) occluding the vessel of a Haversian canal of cortical bone.

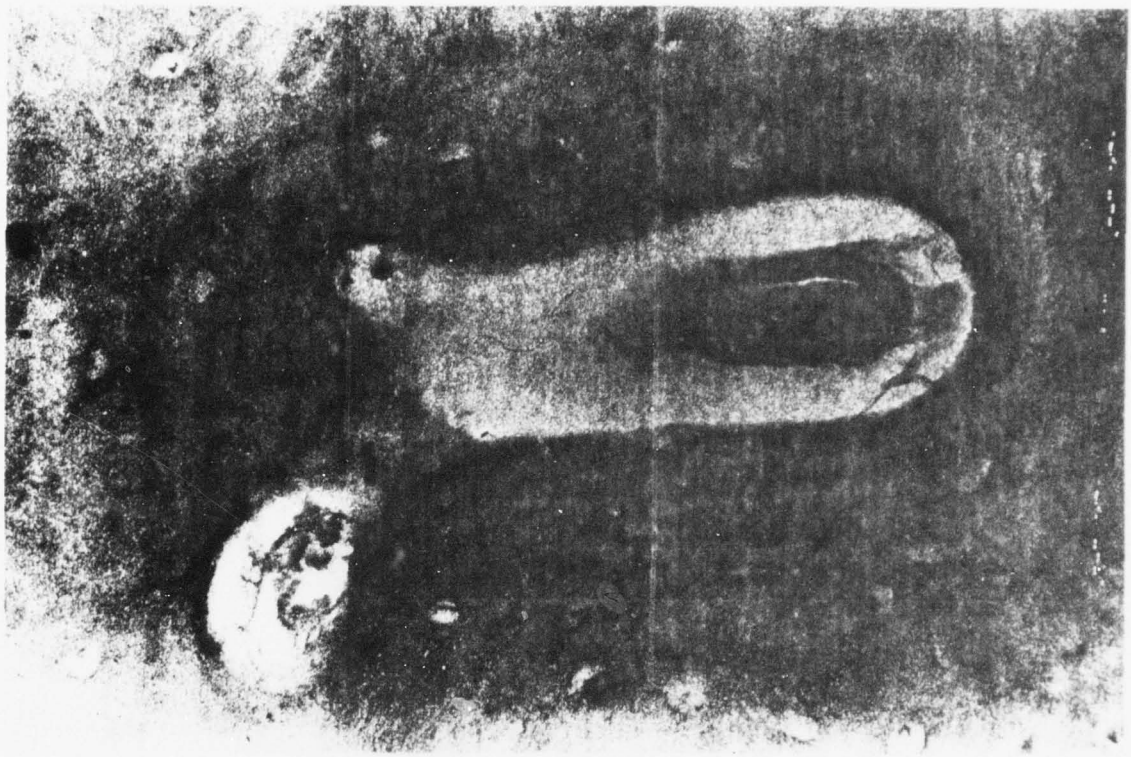


Figure 2 A widened Haversian system with new bone (1) being deposited on its inner surface, and vessel thrombosis (2).

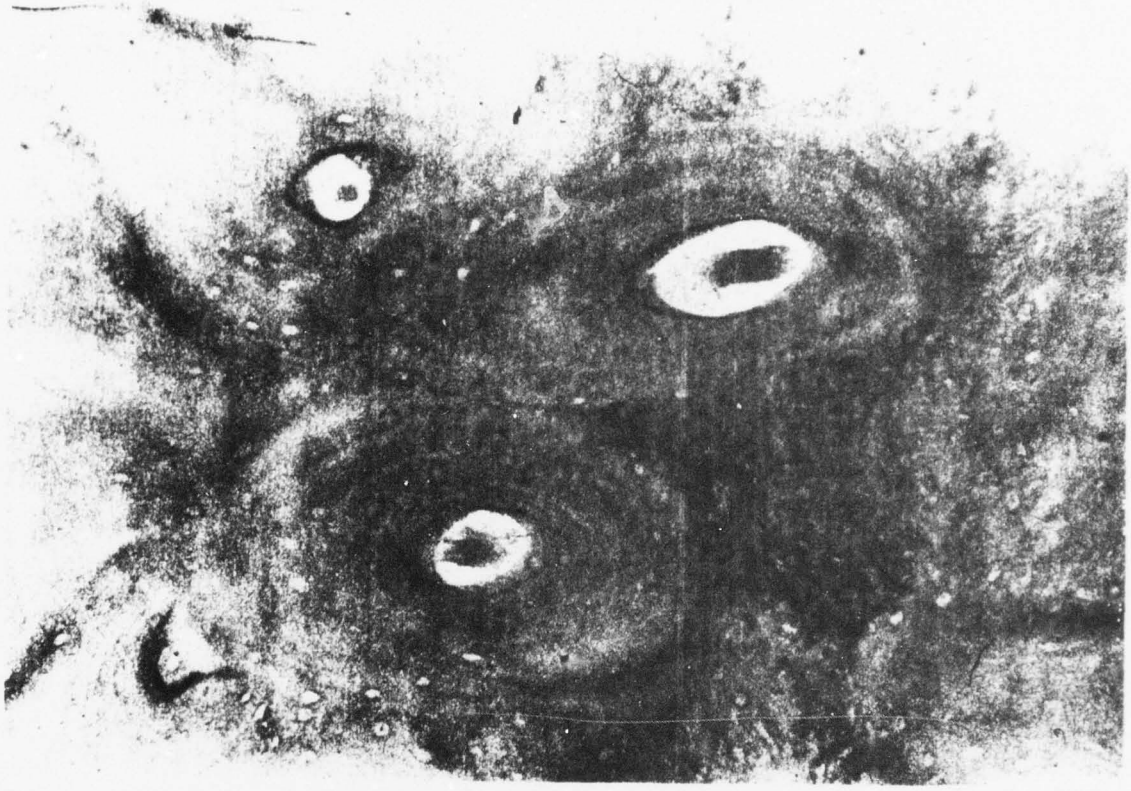


Figure 3 Multiple thrombosed vessels in area of bone necrosis.

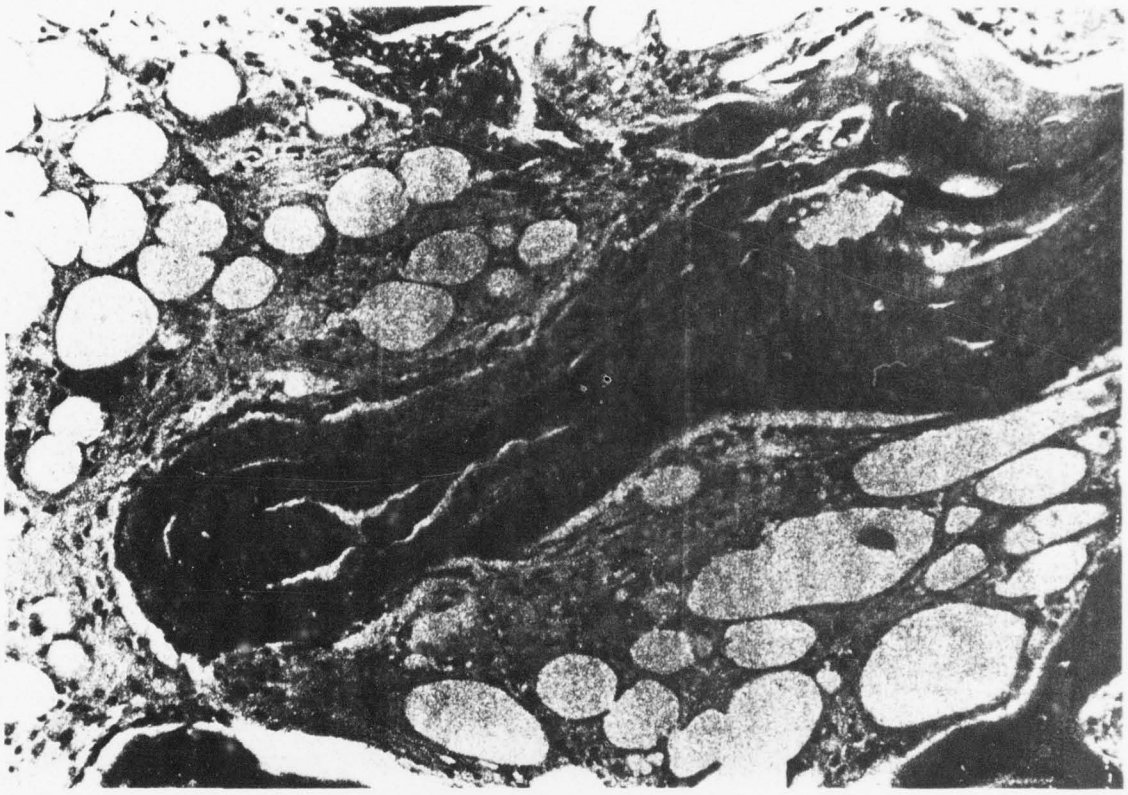


Figure 4 Extensive reparative attempt of bone with appositional new bone (1) formation on necrotic trabecular bone (2) and irregular and wavy cement lines (3).

Within the tubular bones was extensive necrosis of cancellous bone and fatty marrow. The latter resulted in lakes of fat surrounded by foamy histiocytes and giant cells (Fig. 5). Necrotic areas showed extensive osteoclastic activity and reactive new bone formation (Fig. 6). Hemorrhage, hemosiderin-laden macrophages and prominent fibrosis were present in the necrotic foci. There were many medium-sized muscular arteries exhibiting concentric intimal thickening and narrowing of lumens as a result of myointimal cell proliferation and accumulation of collagen (Fig. 7, 8). The endothelial cells of the affected arteries were inordinately prominent.

Bony trabeculae revealed abnormal cement lines, characterized by numerous cement lines intersecting at sharp angles and scalloping patterns (Fig. 9, 10, 11). Occasional osteoclastic and osteoblastic (osteoplastic) activities were noted.

The epiphyseal plate invariably showed foci of necrosis accompanied by fraying of cartilage and calcification (Fig. 12). Occasional hyaline thrombi in capillaries were appreciated.

In one section thinning of the articular bone plate was noted with the articular cartilage overlying the plate also showing a decrease in thickness. Distortion of the collagen framework was apparent when examined by polarizing light.

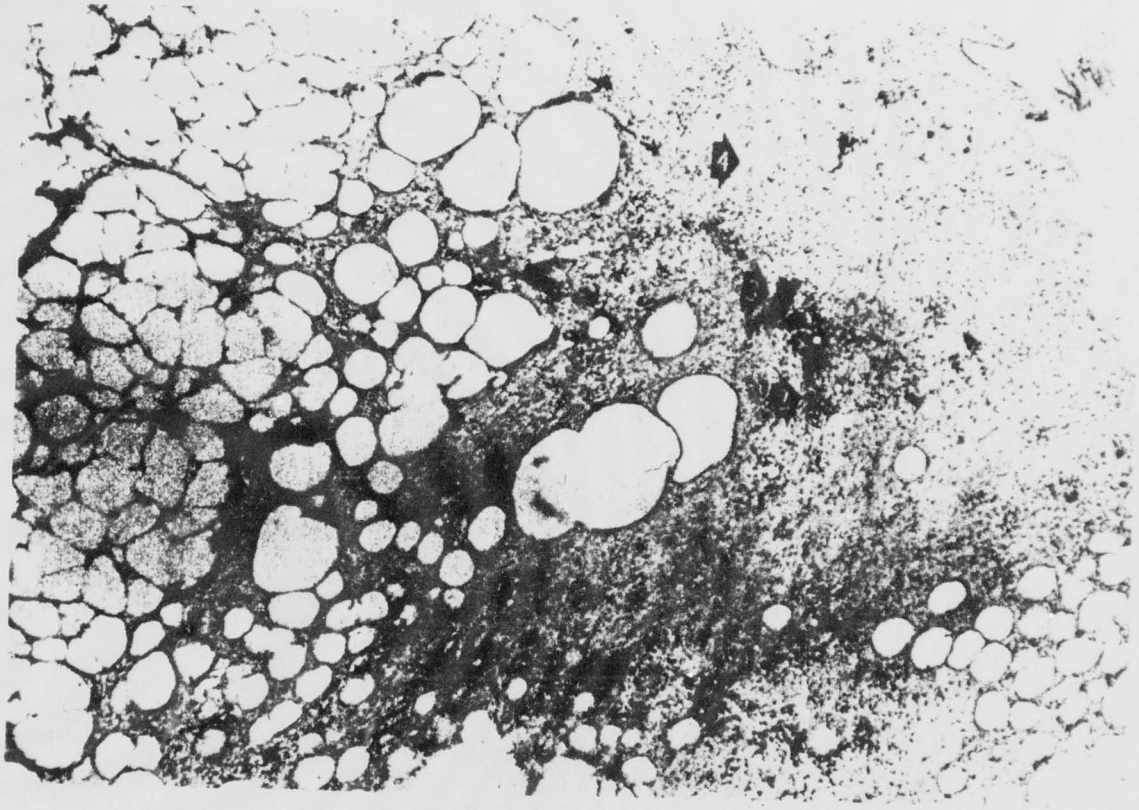



Figure 5 Extensive necrosis of fatty marrow with degenerating fat cells forming lakes of fat (1); hemorrhage (2); hemosiderin-laden macrophages (3); and fibrosis (4) in necrotic foci.

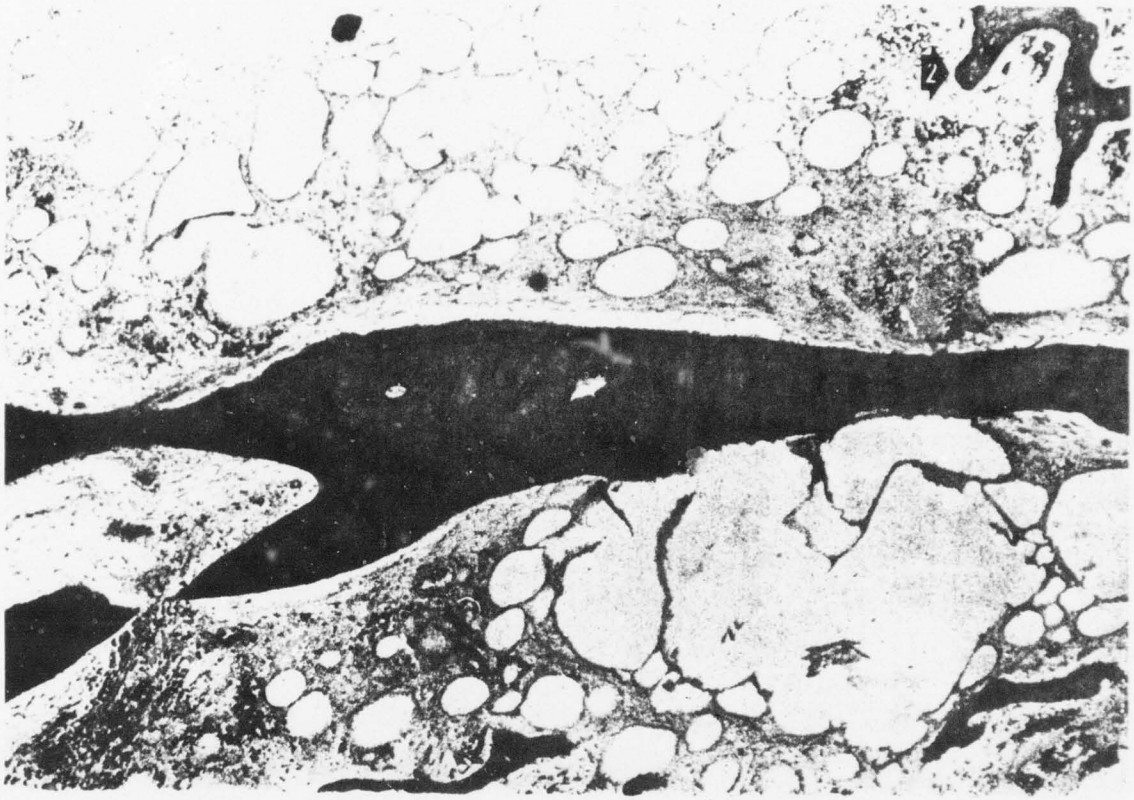



Figure 6 Osteoclastic removal of dead bone (1), osteoblastic activity and new woven (pre-lamellar) bone (2).

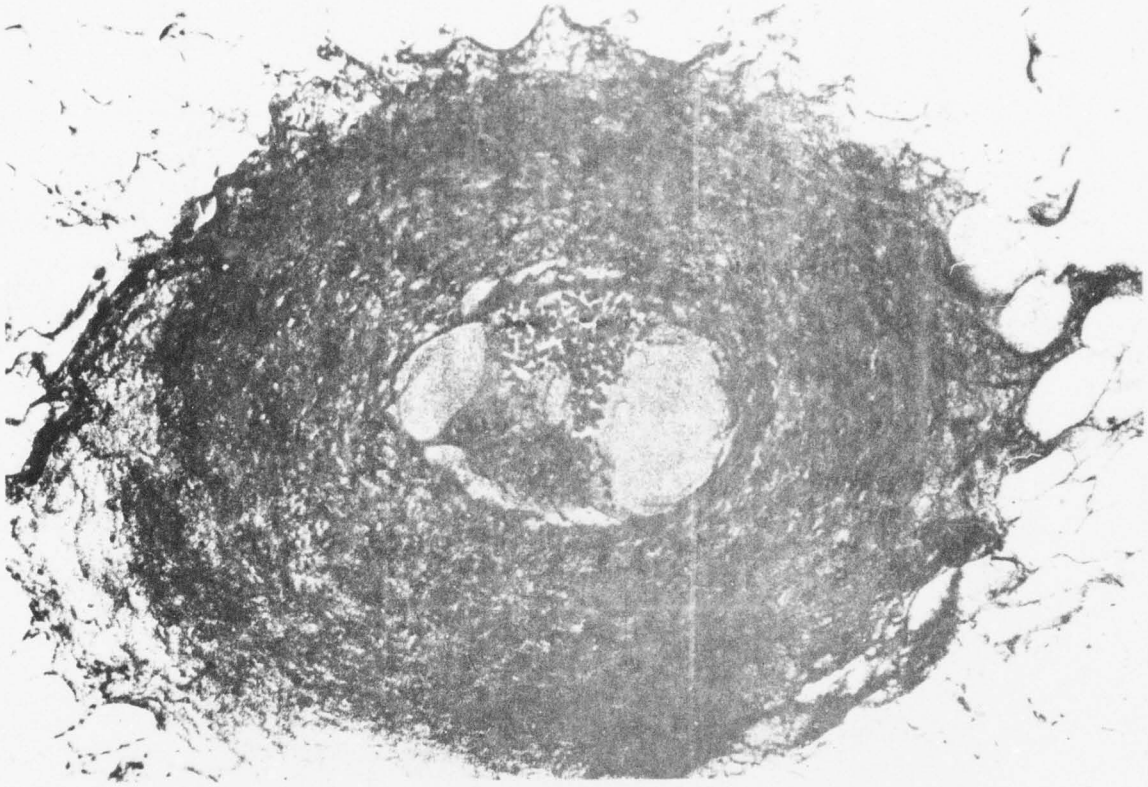


Figure 7 Arterial intimal thickening (arrow) in a transverse section of bone.

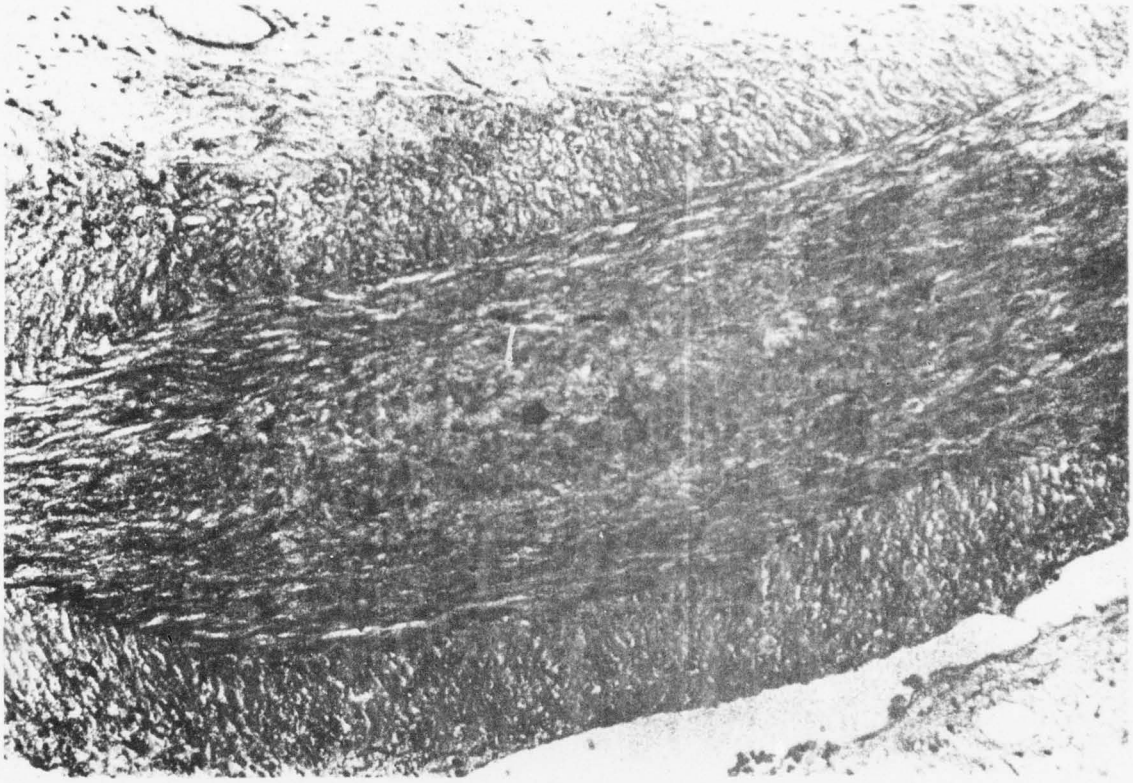


Figure 8 Myointimal cell proliferation (arrow) obliterating lumen of bone vessel.

Figure 9 Abnormal cement lines (arrows) in trabecular bone as the result of injury.

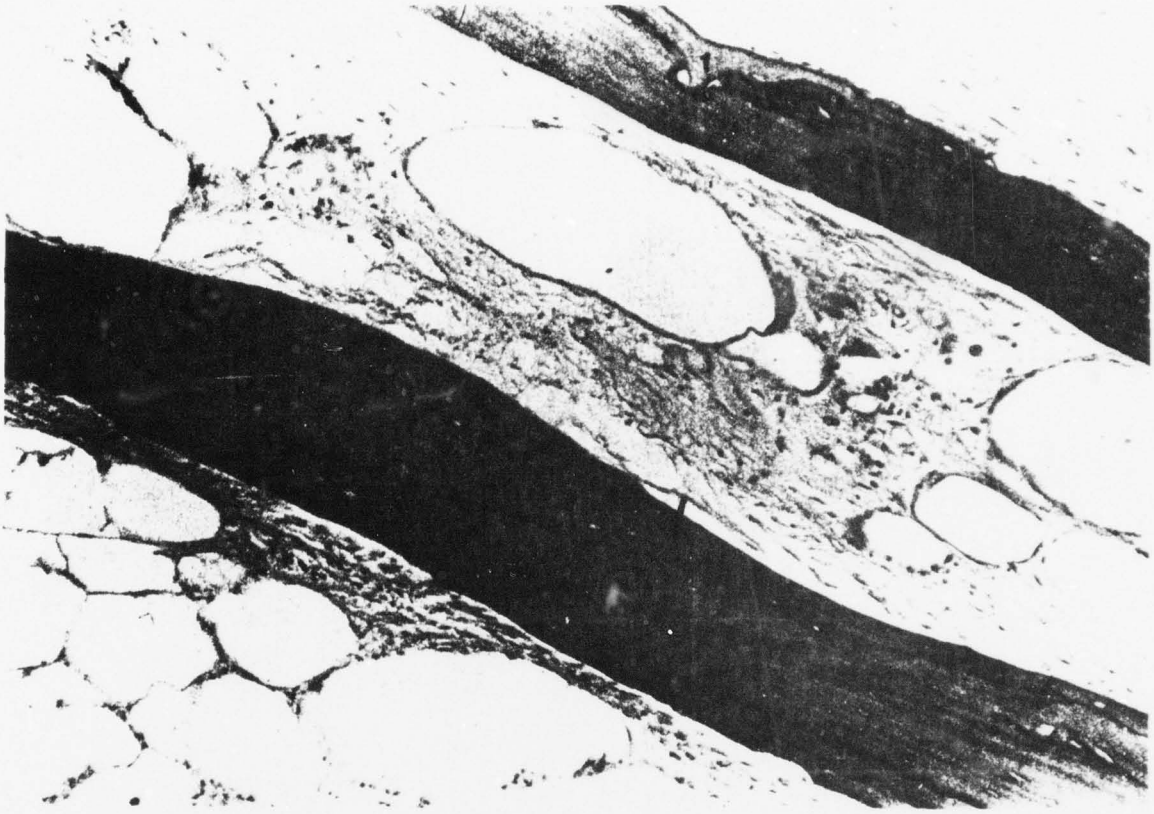


Figure 10 New bone formation following osteoclastic removal of necrotic bone (1), and abnormal cement lines (arrows).

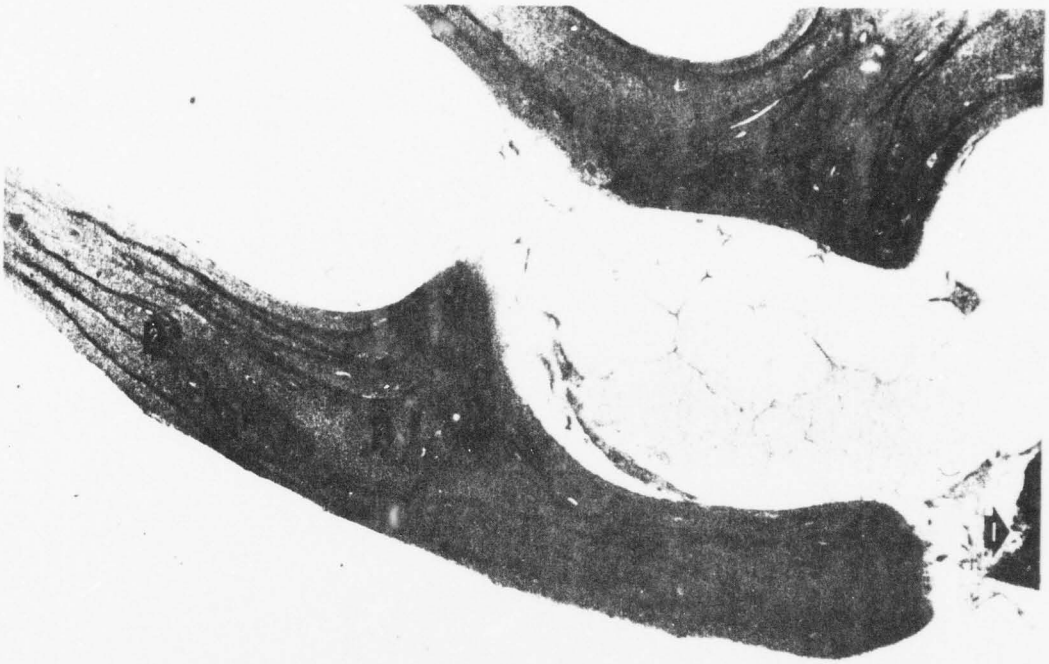


Figure 11 Active dead bone removed by osteoclasts (1), and irregular cement lines (2).

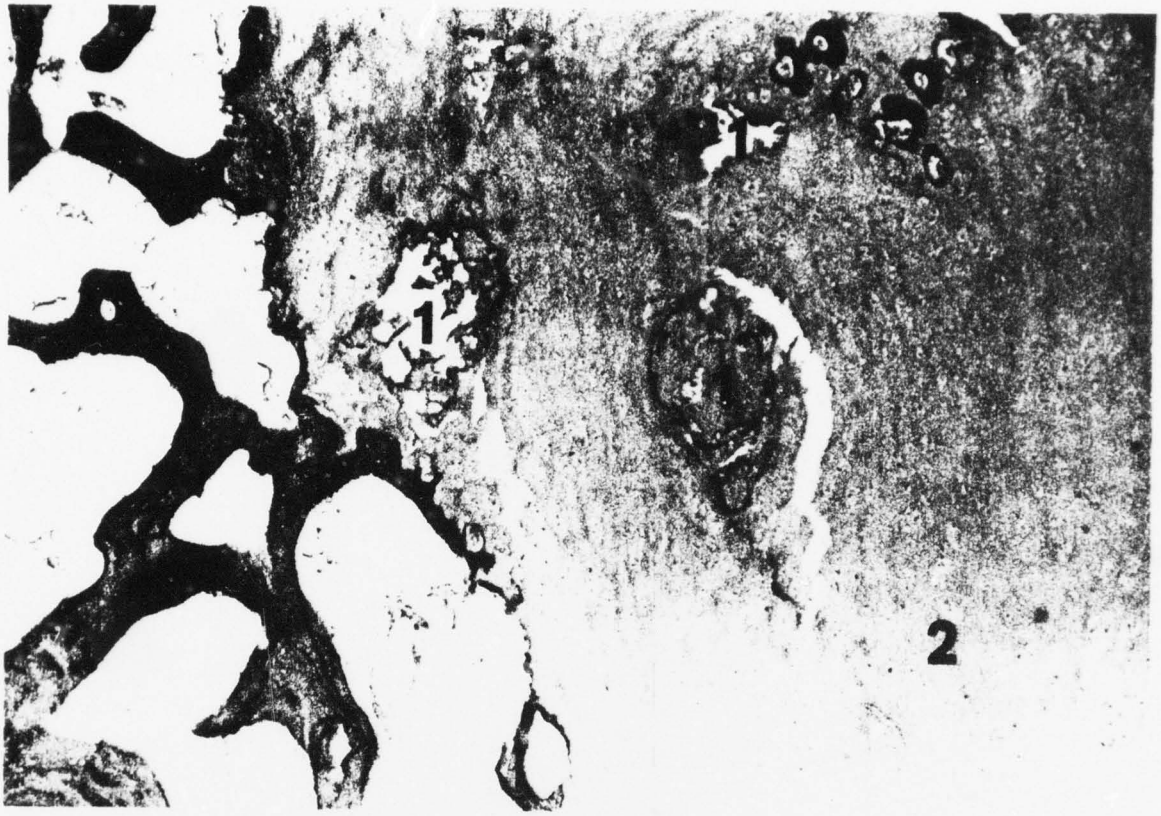



Figure 12 Large areas of necrosis (1) in epiphyseal plate (2) of femoral head.

C. Individual Pathological Reports on Animals with Multiple Exposures

Pig # 1

Diving History: 60 fsw/6 hrs - 79 dives

Treatment: none

Time since last dive: 1 year

Radiography: Changes consistent with aseptic bone necrosis in femurs and humeri.

Histopathology: The bone marrow in the metaphysis shows extensive necrosis of fat and trabecular bones. There are lakes of fat surrounded by foreign body-type giant cells, foamy histiocytes, hemosiderin-laden macrophages, and extensive fibrosis. Interspersed between fibrotic areas are newly formed small trabecular bones, which are "woven" in character. Sequestrum surrounded by new appositional bone is also present. The adjacent cortical bone reveals widened Haversian canals with new bone formation. Medium-sized muscular arteries show concentric uniform thickening of the intima due to myointimal cell proliferation. Some of them contain hyaline thrombi.

The hyaline thrombi are also noted in the capillaries of the articular bone plate. This is associated with areas of new bone formation. The articular cartilage remains intact.

There is focal necrosis of the epiphyseal plate.

Summary: (1) Extensive marrow and trabecular bone necrosis, associated with reactive new trabecular bone formation. (2) Hyaline thrombi in capillaries and medium-sized muscular arteries and thickening of the intima in the latter.

Pig # 40

Diving History: 60 fsw/6 hrs - 40 dives

Treatment: none

Time since last dive: 8 months

Radiography: Changes consistent with early aseptic bone necrosis in right humerus, left and right femurs

Histopathology: The fatty bone marrow exhibits fat necrosis, foamy histiocytes, hemosiderin-laden macrophages and fibrosis in the metaphysis. Small arteries in the adjacent areas show moderate intimal thickening. Focal peritrabecular new bone formation is noted. The epiphyseal plate is thin, with many segments replaced by bone. There are hyaline thrombi in capillaries in subarticular bone plate. However, the articular cartilage and bone plate appear unscathed.

Summary: (1) Fatty bone marrow necrosis and fibrosis. (2) Hyaline thrombi in capillaries. (3) Bone unscathed. (4) Unable to evaluate significance of changes in epiphyseal plate at this time.

Pig # 42

Diving History: 60 fsw/6 hrs - 78 dives

Treatment: none

Time since last dive: 11 months

Radiography: Not remarkable (14 months previous to sacrifice left humeri was thought to show early infarct but report 1 month later was negative).

Histopathology: There is extensive replacement of normal

fatty marrow and trabecular bone by fibrous tissue in the metaphysis. Occasional dead trabecular bones are entrapped within the fibrous tissue. The cortical bone is intact in the metaphysis. There is remarkable alteration of articular cartilage and underlying bone plate in the femoral head. The changes are characterized by hyaline thrombi in capillaries and thinning of the articular bone plate with occasional osteoplastic activity. The articular cartilage overlying the attenuated articular bone plate shows decrease in thickness (Fig. 13). Distortion of cartilage collagen framework is apparent when the section is examined with a polarizing light microscope.

Focal necrosis and disruption of epiphyseal plate is also noted. Cross sections of diaphysis of right femur demonstrate subperiosteal new bone formation, one-third to one-half of the thickness of the original cortical bone. Mild subendosteal new bone is also present. The great majority of osteocytes of the original cortical bone have disappeared from their respective lacunae. Their associated lamellar bone has lost normal lamellar structure (Fig. 14), and collagen fibers appear disorganized as revealed by polarizing light microscopy (Fig. 15). Within the presumably dead cortical bone are occasional newly formed osteons containing normal appearing osteocytes and lamellar bone. Episodes of remodeling are evident as revealed by eccentric cement lines. It is important to point out that the Haversian canals are almost empty in the necrotic cortical bone (Fig. 16).

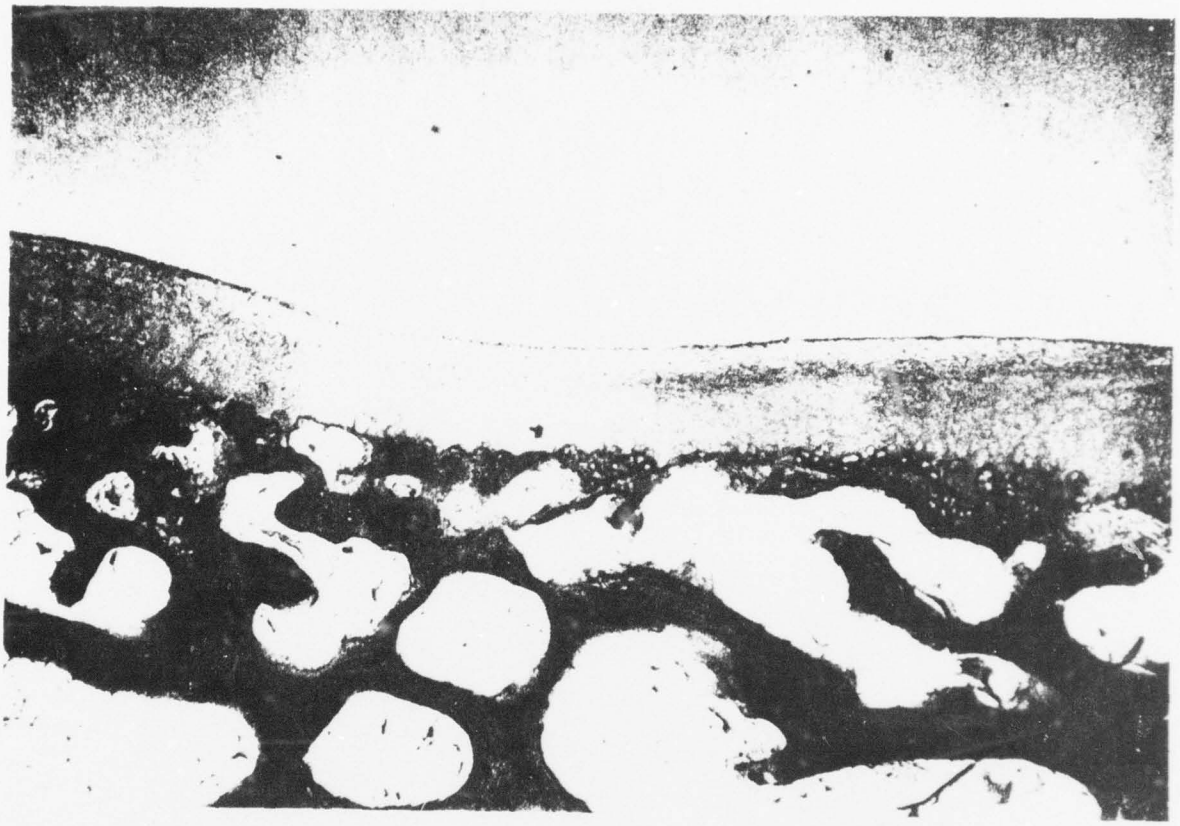


Figure 13 Articular cartilage overlying attenuated articular bone plate.

Figure 14 Lamellar bone showing loss of normal structure.

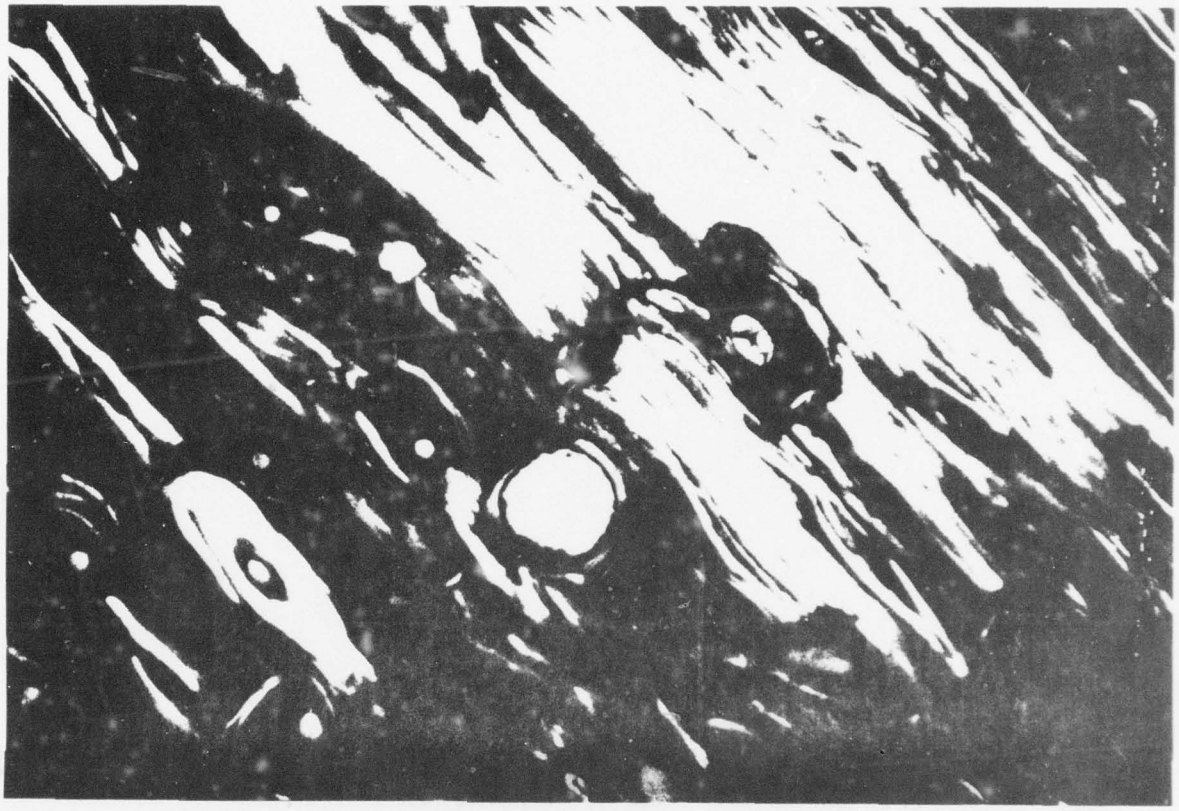


Figure 15 Collagen fibers appearing disorganized as revealed by polarizing light microscopy.

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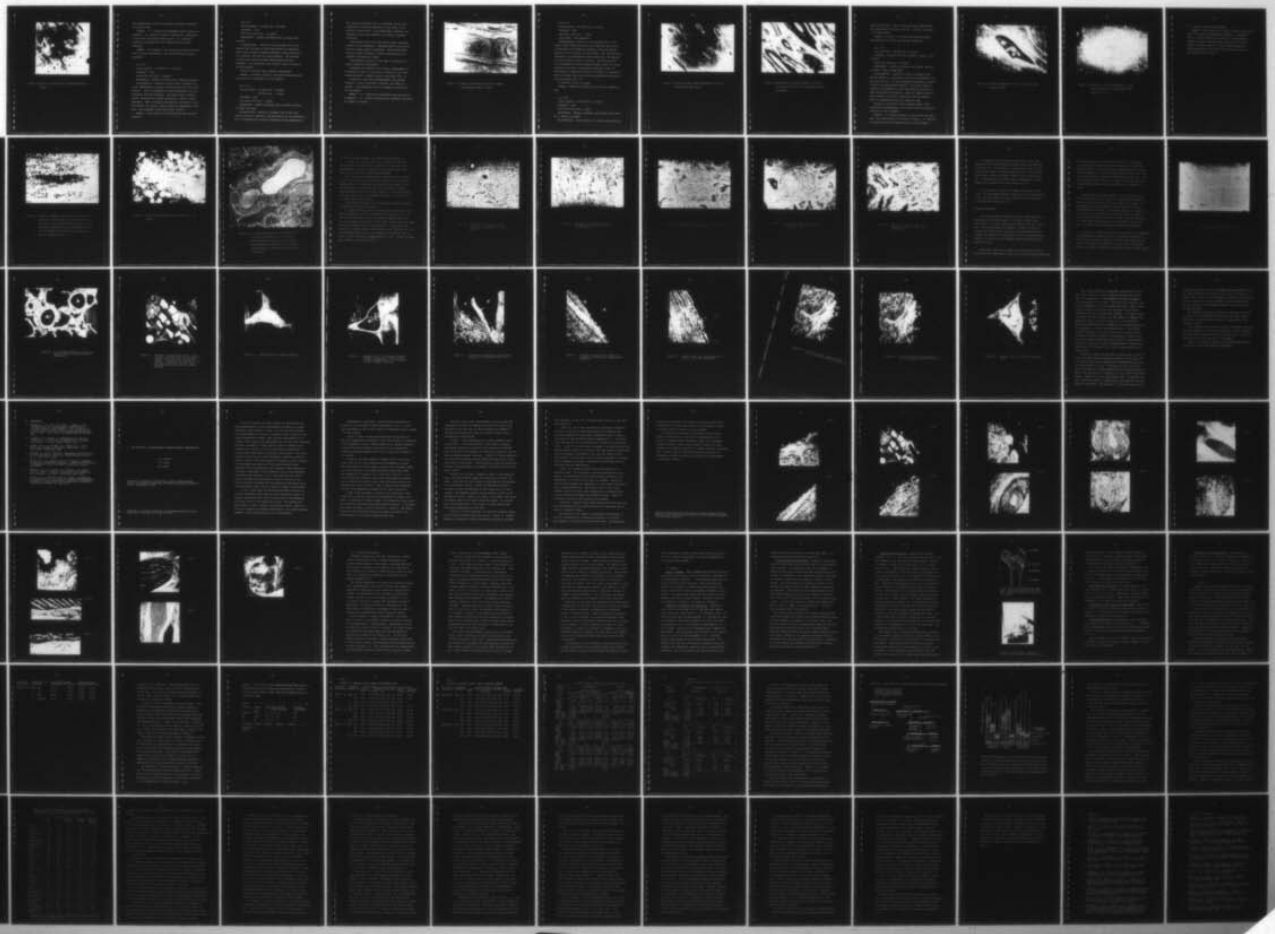

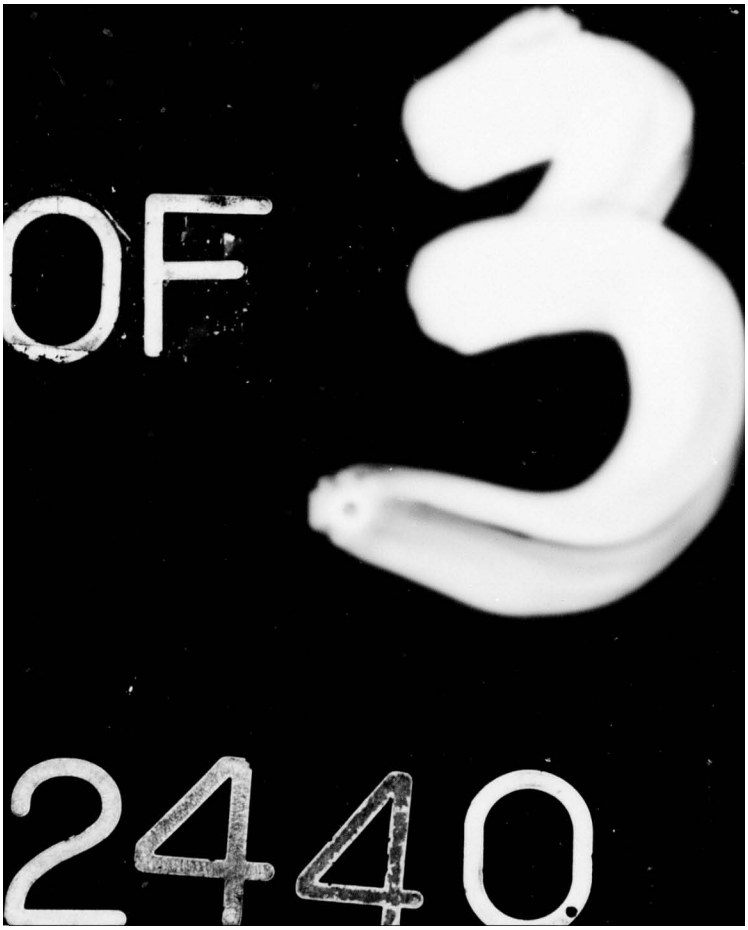
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Figure 16 Necrotic cortical bone with empty Haversian canals.

The subperiosteal bone has the normal histologic appearance of cortical bone.

Summary: (1) Fibrosis of metaphyseal marrow cavity and necrosis. (2) Hyaline thrombi in capillaries, attenuation of articular cartilage and bone plate. (3) Extensive necrosis of cortical bone with subperiosteal new bone formation.

Comment: The changes in the articular cartilage and bone plate are very interesting. The lesion should be further evaluated.

Pig # 58

Diving History: 60 fsw/6 hrs - 35 dives

Treatment: none

Time since last dive: 2 months

Radiography: Changes consistent with avascular necrosis

Histopathology: Sections of right femur (proximal end) reveal derangement of osteons in the cortical bone with focal necrosis. This is accompanied by both subendosteal and subperiosteal new bone formation. The marrow cavity contains several arteries with intimal thickening as noted in other specimens. There is hyaline-like material infiltrate in the bone marrow, the nature of which cannot be determined at this time. The epiphyseal plate shows focal necrosis.

Summary: Focal necrosis of cortical bone with new bone formation.

Pig # 80

Diving History: 60 fsw/6 hrs - 80 dives

Treatment: none

Time since last dive: 11 months.

Radiography: Changes consistent with avascular bone necrosis.

Histopathology: Sections of the proximal end of left humerus show necrosis of fatty bone marrow and trabecular bone, which provokes extensive fibrosis, foreign body giant cell reaction, and mild new bone formation. The adjacent cortical bone exhibits marked subperiosteal new bone formation. The epiphyseal plate contains areas of necrosis and obliteration.

Section of proximal femur appears unremarkable.

Summary: Necrosis, fibrosis, and foreign body granuloma-type reaction of metaphysis of left humerus.

Pig # 117

Diving History: 60 fsw/6 hrs - 34 dives
80 fsw/4 hrs - 2 dives

Treatment: none

Time since last dive: 3 weeks

Radiography: Changes consistent with avascular necrosis in femurs and humeri

Histopathology: Section of proximal end of left femur reveals extensive fibrosis in the bone marrow of the metaphysis. This is accompanied by numerous hemosiderin-laden macrophages.

The necrotic trabecular bone is surrounded by new bone. Subperiosteal new bone formation is also noted in the adjacent cortical bone. Focal hemorrhage, fibrosis, and trabecular woven bone formation are present in greater trochanter.

The proximal end of the left humerus shows extensive fibrosis in the metaphysis. Entrapped within the fibrous tissue are many necrotic trabeculae. Many medium-sized arteries exhibit concentric intimal proliferation with consequent narrowing of lumens.

The epiphyseal plates of both femur and humerus are almost completely obliterated.

Concentric thickening of the intima, predominantly by collagenous tissue, is also seen in arteries in the soft tissue adjacent to femoral neck of right femur (Fig. 17).

Sections of lung show bronchopneumonia and abscess formation. Focal atelectasis, fibrosis, and edema are present. The endothelial cells of pulmonary arteries are very prominent.

Summary: (1) Fibrosis and trabecular bone necrosis of metaphysis. (2) Intimal thickening and consequent narrowing of lumens in arteries.

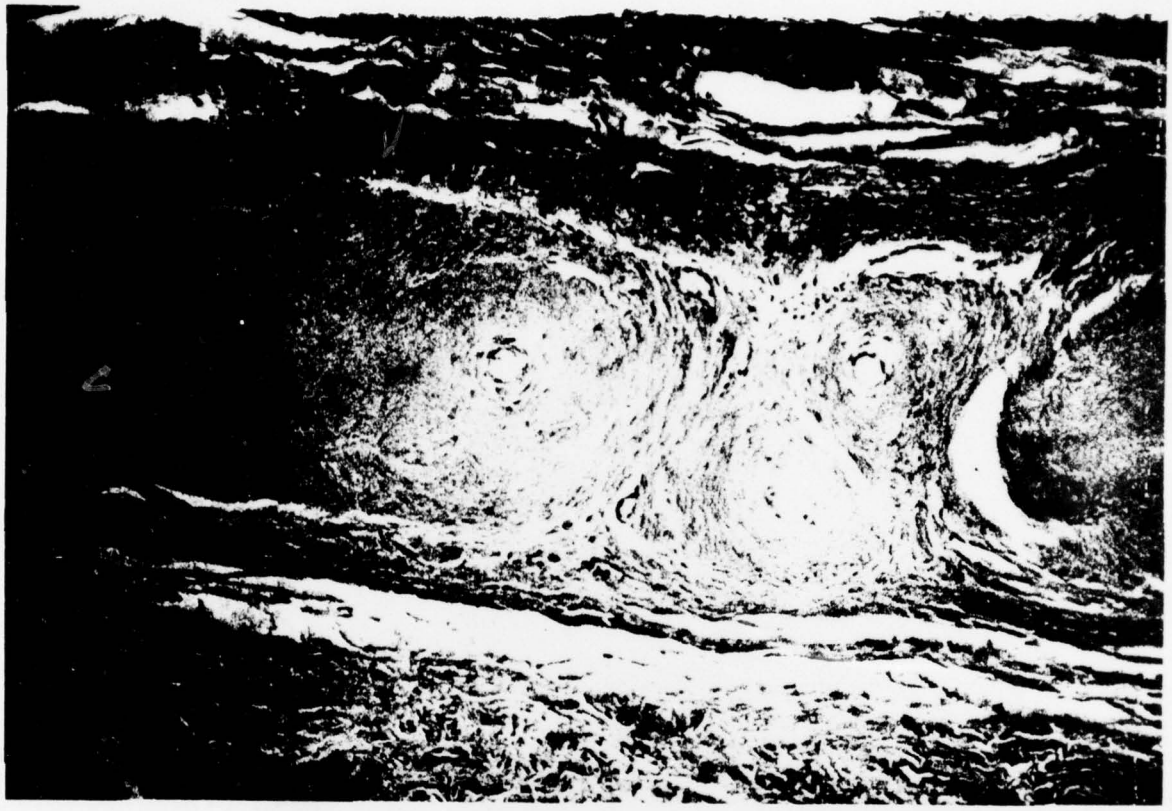

Figure 17 Concentric thickening of arterial intima in extraosseous femoral tissue.

Pig # 118

Diving History: 60 fsw/6 hrs - 10 dives

Treatment: none

Time since last dive: 4 days

Radiography: Not remarkable

Histopathology: Section of the proximal end of left humerus shows extensive necrosis of trabecular bone, which is associated with extensive fibrosis of the bone marrow. Evidence of fat necrosis is also present. The necrotic bone trabeculae are surrounded by rims of new bone. The epiphyseal plate contains areas of necrosis. The cortical bone in the metaphysis reveals deranged osteons, which are widely separated by necrotic interstitial bone plate (Fig. 18). Derangement of lamellar structure of collagen in the necrotic bone was evident when the section was examined by polarizing light microscopy (Fig. 19).

No vascular injury is evident.

Summary: Extensive necrosis of cortical and trabecular bone.

Pig # 200

Diving History: 60 fsw/6 hrs - 35 dives

Treatment: none

Time since last dive: 1 year

Radiography: Changes consistent with aseptic bone necrosis in femurs and humeri.

Histopathology: Focal necrosis of cortical bone and bone

Figure 18 Deranged osteons widely separated by necrotic interstitial bone plate.

Figure 19 Derangement of collagen lamellar structure in necrotic bone as seen by polarizing light microscopy.

marrow are present. This is associated with subendosteal and subperiosteal new bone formation. Fibrosis is present in the bone marrow.

Summary: Necrosis of cortical bone and bone marrow associated with fibrosis and new bone formation.

Pig # 106

Diving History: 60 fsw/6 hrs - 8 dives

Treatment: Platelet function inhibitor, aspirin, anti-coagulant

Time since last dive: 48 hours

Radiography: Changes consistent with aseptic necrosis in humeri; femurs unremarkable

Histopathology: Sections of femur and humerus show hyaline thrombi in capillaries (Fig. 20) and myointimal cell proliferation (Fig. 21) with consequent narrowing of lumens of small and medium-sized arteries. The latter change is frequently accompanied by focal perivascular collection of foamy histiocytes and fibrosis. Areas of appositional new bone formation on necrotic trabecular bone are apparent. There is subperiosteal new bone formation also.

Section of lung shows severe pulmonary edema, focal atelectasis and hemorrhage. The intimal cells of pulmonary arteries are very prominent.

Summary: (1) Hyaline thrombi in capillaries and myointimal cell proliferation of arteries in bones. (2) Necrosis of trabecular bone with appositional new bone formation.

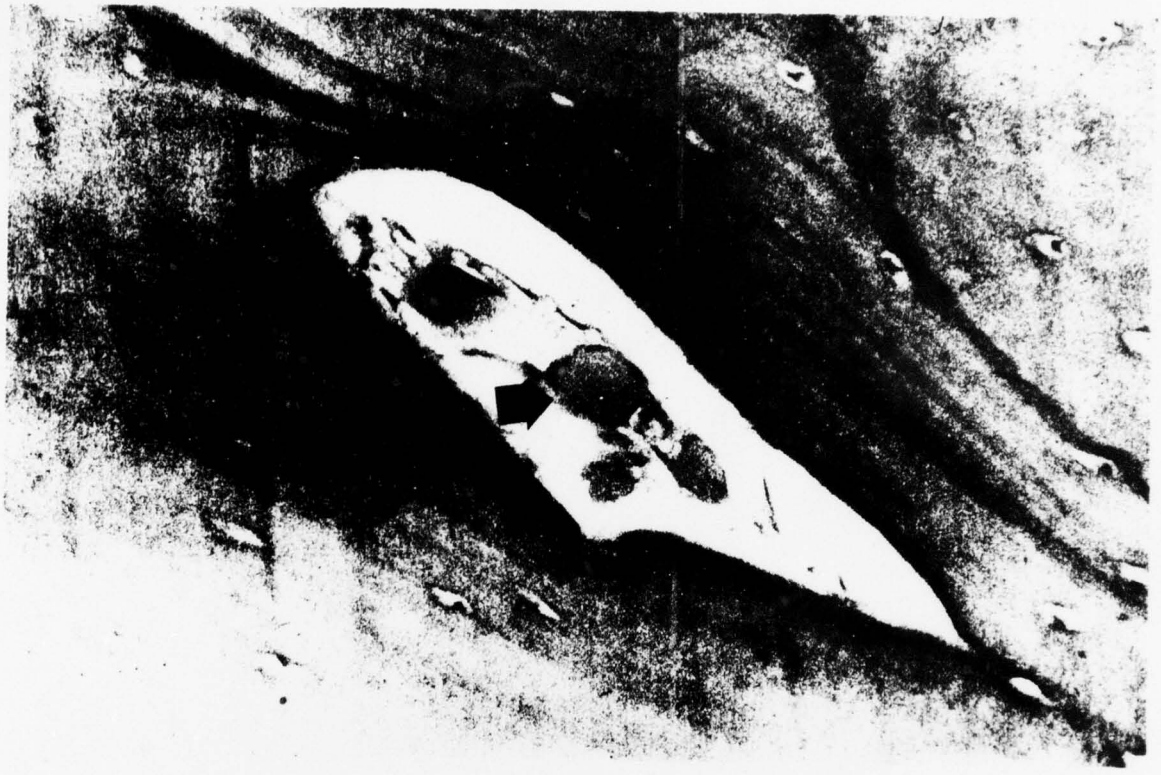

Figure 20 Hyaline thrombosis (arrow) in the acute phase of bone changes.

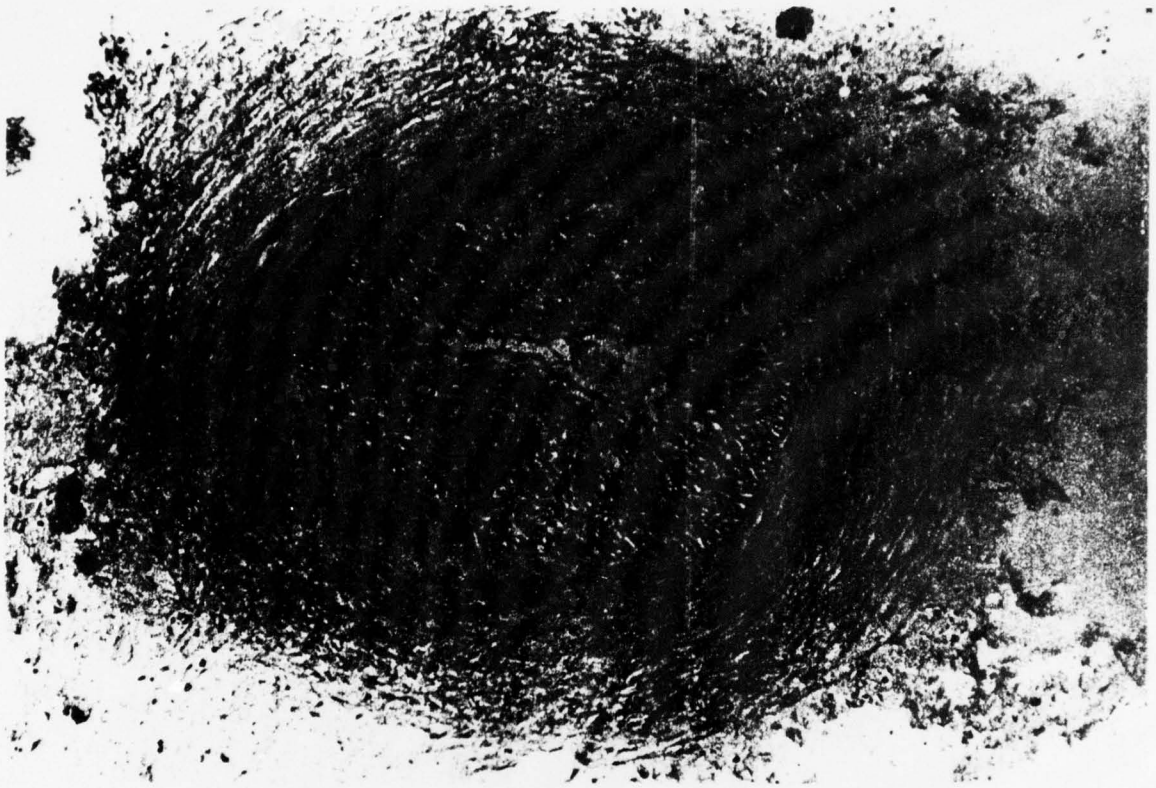

Figure 21 Concentric intimal thickening (1) with lumen narrowing (2) and prominent endothelial cells (3).

(3) Pulmonary edema and hemorrhage.

Comment: This animal at the time of sacrifice manifested signs of severe CNS decompression sickness. It had been part of a study evaluating the ability of antithrombotic therapy to prevent osteonecrosis, and had been receiving a platelet function inhibitor, aspirin and anticoagulant. Gross examination of the spinal cord revealed an area of hemorrhage at the C-5 level.

Individual Pathological Reports on Animals with Single Exposures

Pig # 116

Diving History: 80 fsw/4 hrs - 1 dive

Treatment: none

Time since last dive: 2 hours

Radiography: Not remarkable

Histopathology: There is no significant alteration of bones. Lungs show severe pulmonary edema, focal atelectasis and hemorrhage.

Pig # 157

Diving History: 80 fsw/4 hrs - 1 dive

Treatment: none

Time since last dive: 4 months

Radiography: not remarkable

Histopathology: Sections of right femur show concentric thickening of intima of intramedullary arteries due to myo-intimal cell proliferation. There are hyaline thrombi in the vessels of Haversian canals. Bone is generally unscathed, except for focal necrosis of the epiphyseal plate. Mild subperiosteal and subendosteal new bone formation is present.

Summmary: (1) Hyaline thrombi in vessels of Haversian canals. (2) Intimal thickening of intramedullary arteries.

D. Endothelial Investigation

To determine the role of the vessel in the coagulation factor consumptive process, the vascular integrity in one control animal with no history of diving, and in one animal exposed to 60 fsw for 6 hours with 30 fpm decompression on four successive days, was studied.

Following surgical exposure and cannulation of the femoral and internal iliac arteries, the vessels were simultaneously perfused and fixed with half-strength Karnovsky's solution under 20 cm $H_2O$ hydrostatic pressure for 20 minutes *in vivo*. After removal from the animal, they were postfixed in osmium tetroxide and epon-embedded for electron microscopic study. The terminal aorta was fixed *in vivo* by the same procedure but was also pressure-perfused with 0.3% silver nitrate solution for histopathologic examination of stained endothelial surfaces. Damaged endothelial surfaces were determined by the lack of staining due to the absence of cells which were sloughed during the injury (Fig. 22). The sloughing process resulted in endothelial cells circulating in the blood as seen in Figure 23.

With the exception of these preliminary observations the study remains incomplete. A segment of the carotid endothelium, obtained and prepared in the same manner, was also readied for assessment of proliferative activity by incubation in $^{3}H$-thymidine for autoradiographic observation of damaged endothelial tissue in the reparative stage. The incorporation of radioactive material is roughly proportional to the extent of endothelial damage.

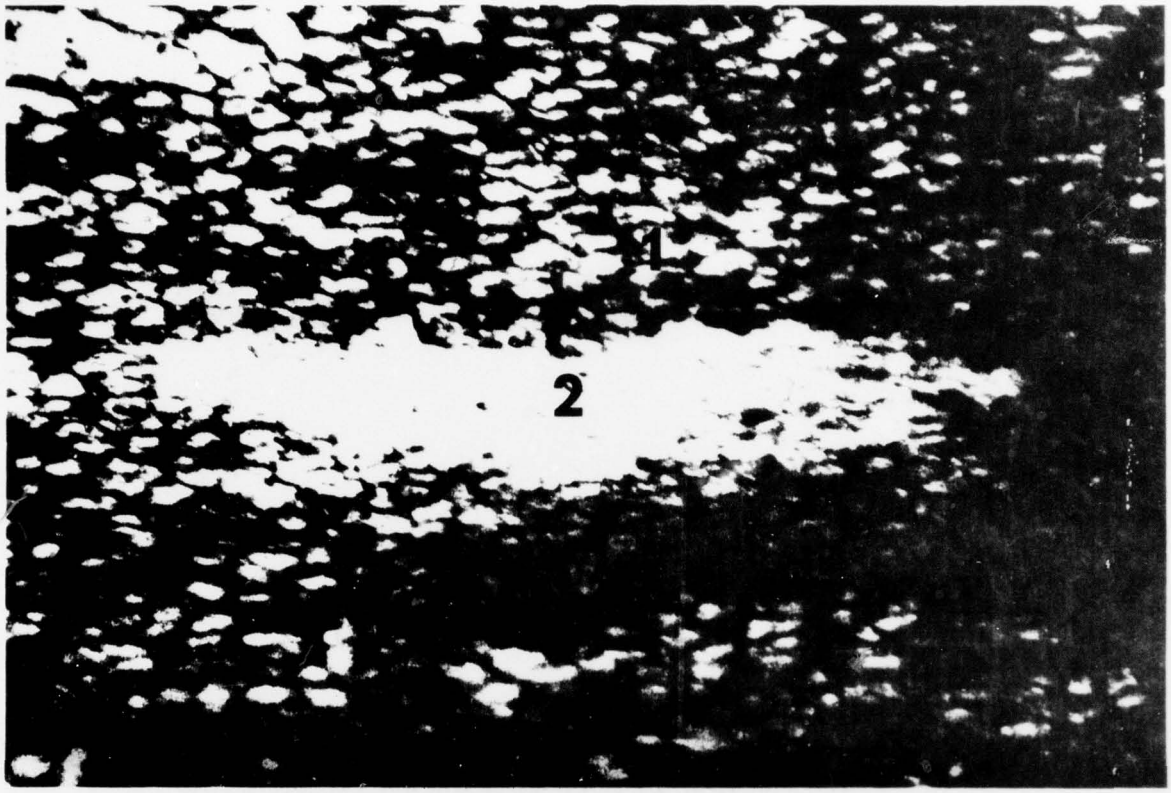


Figure 22 Section of terminal aorta after an intravenous injection of silver stain to outline endothelial juctures, with endothelialized area of the vessel possessing characteristically pavemented pattern (1) and endothelial cells having sloughed in an area of vascular injury (2).

Figure 23 Circulating endothelial cells (arrow) in blood sample.

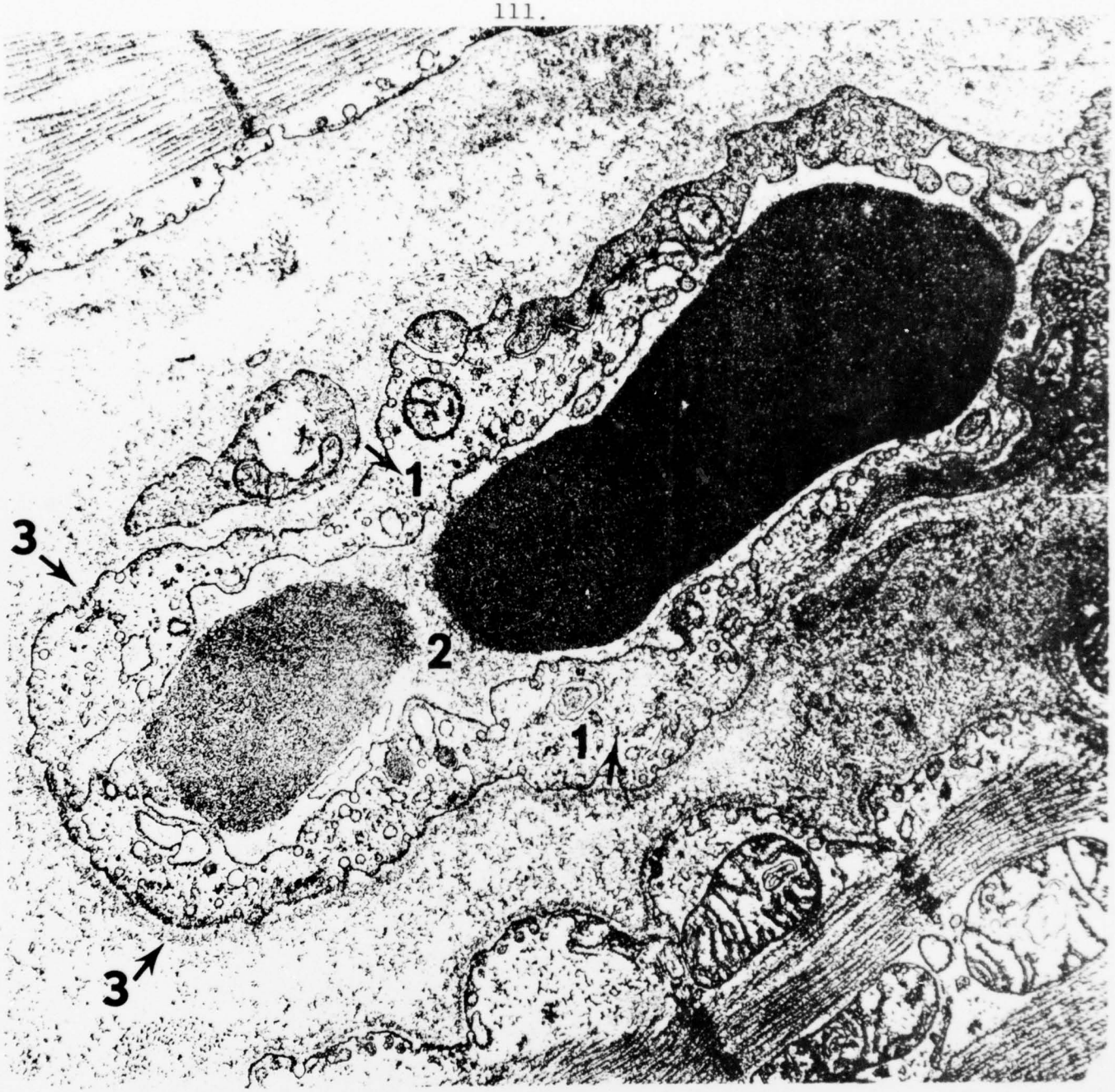


Figure 24 An electron micrograph of endothelial cells (1) lining the lumen (2) of a capillary. When the basement membrane (3) is exposed by sloughing of endothelial cells following vascular injury, the coagulation mechanism is activated.

E. Acute Cellular Response to Inadequate Decompression

To answer the question what acute changes resulted from the exposure, four animals were exposed to 60 fsw/6 hrs. and decompressed at 30 fpm. Animals were then sacrificed at one, two, four and 26 hours following decompression.

None of the pigs exhibited signs of decompression sickness by the time of sacrifice although one animal (at 4 hours after decompression) had remarkable and extensive subcutaneous capillary hemorrhage. Previous studies had indicated that demonstrable changes could be found most readily in lungs and kidneys; therefore, these organs were sampled in detail. No gross signs of tissue damage were present.

Tissues were prepared for both light and electron microscopy by techniques detailed in Section 3.

In kidney specimens examined from the pig sacrificed one hour after decompression, only a rare bubble was seen (Fig. 25). In the animal sacrificed at four hours numerous bubbles were seen in the peritubular capillaries of the cortex (Figure 26) by 26 hours, however, the bubbles were no longer present. In the two, four, and 26 hour animals, thrombi were present in the glomeruli (Fig. 27) and peritubular capillaries; and glomerular necrosis was seen as early as four hours after decompression (Fig. 28). Tubular necrosis was evident at 26 hours (Fig. 29).

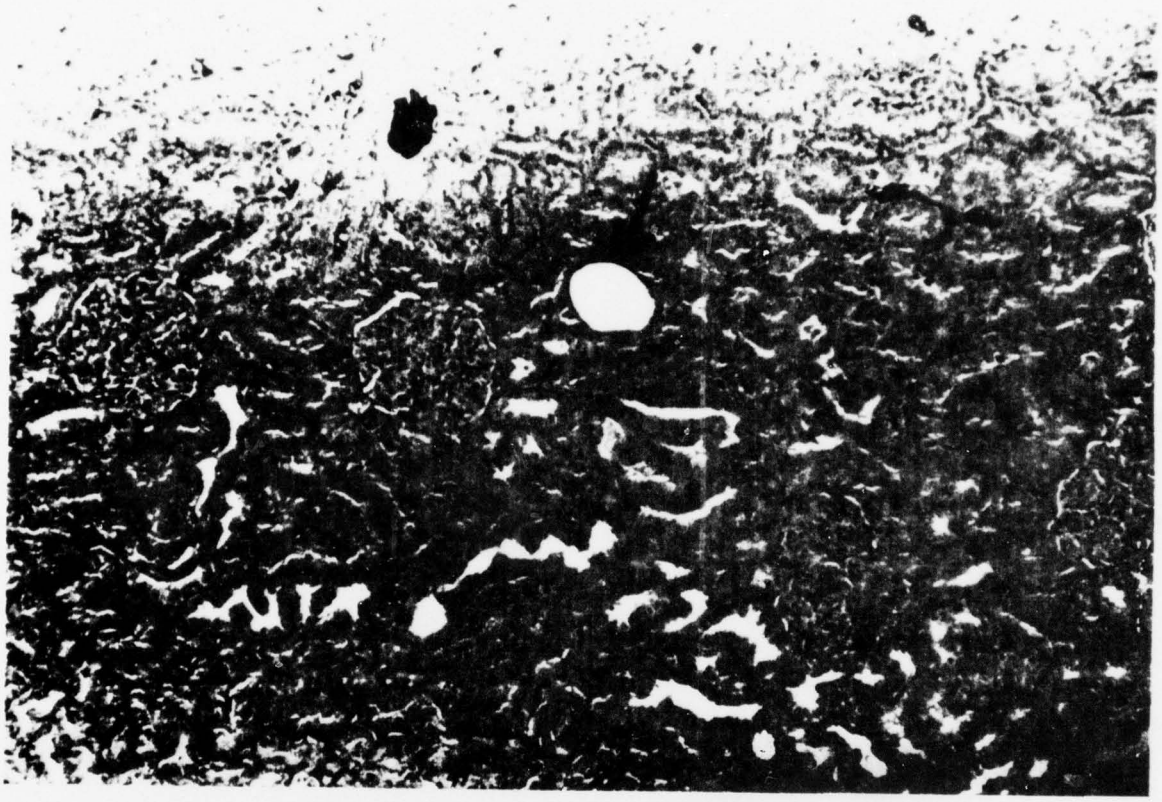

Figure 25- A rare air bubble (arrow) found one hour after decompression in the kidney. Glomeruli (G) appear normal.

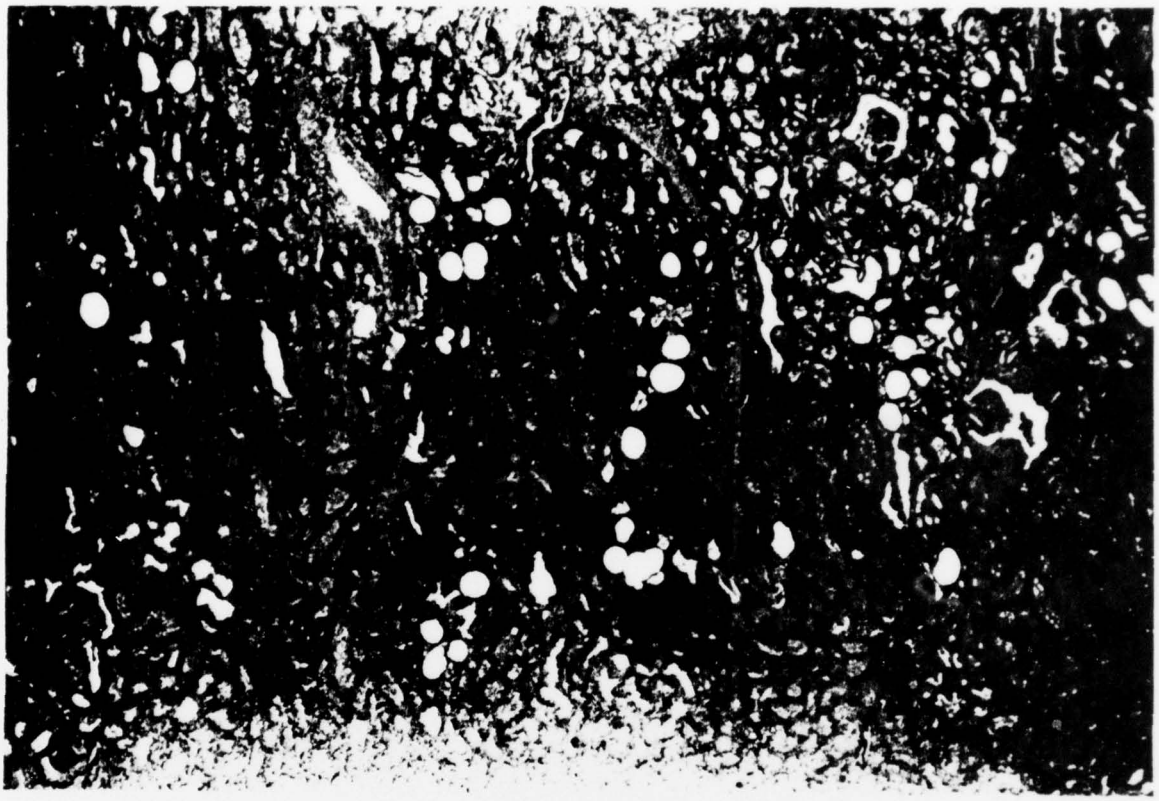

Figure 26 Numerous air bubbles (arrows) seen at four hours after decompression in the kidney

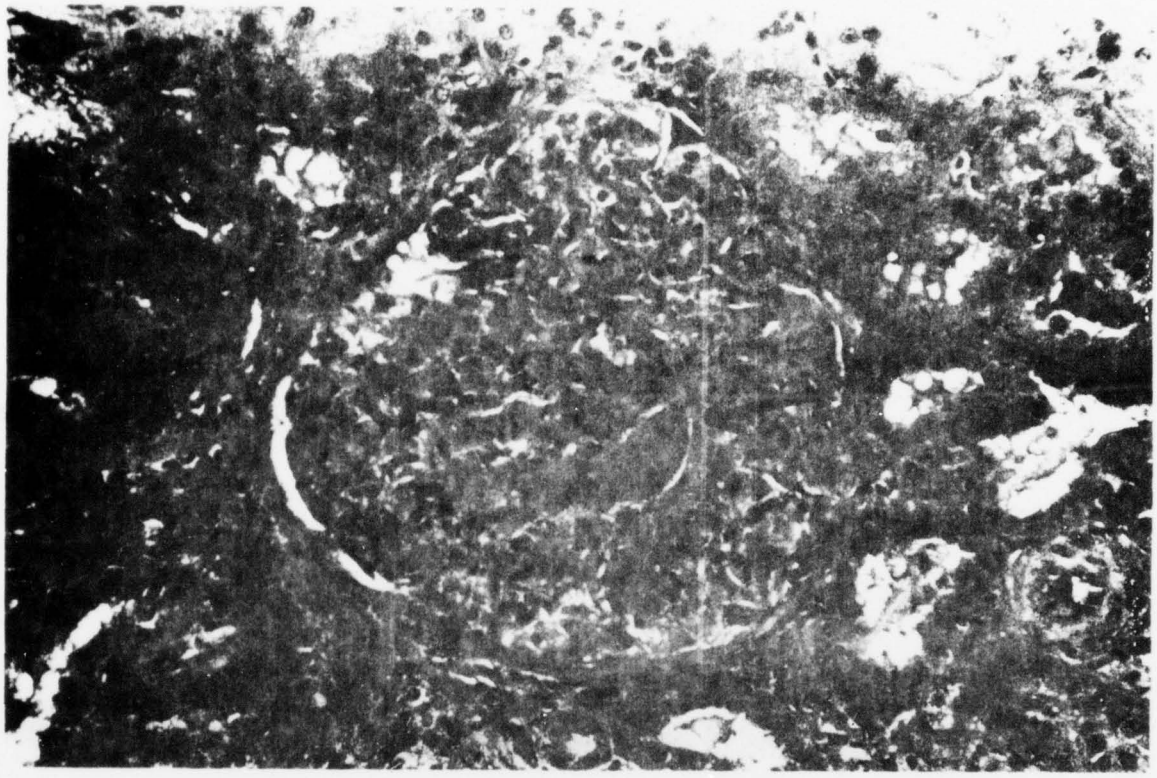

Figure 27- Thrombus (arrow) seen in a glomerulus (G) four after decompression.

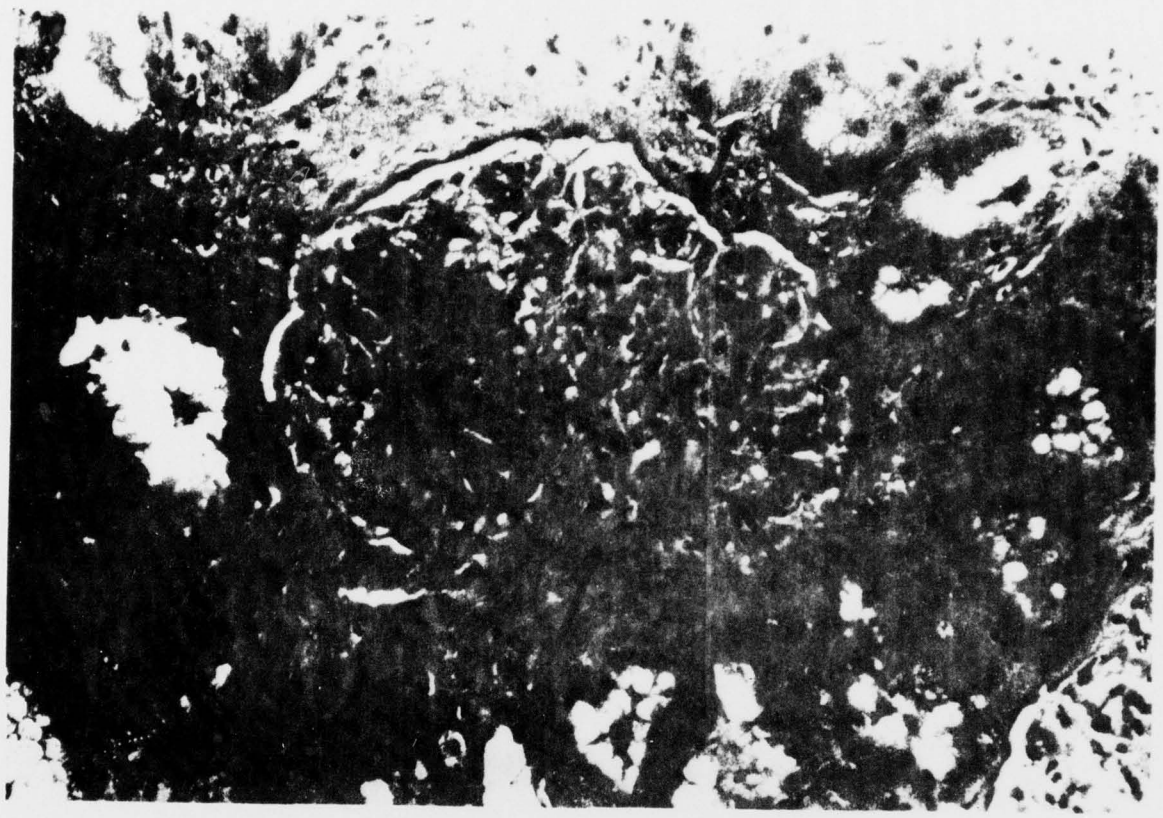

Figure 28 Necrosis (arrows) seen at four hours following decompression in a glomerulus (G).

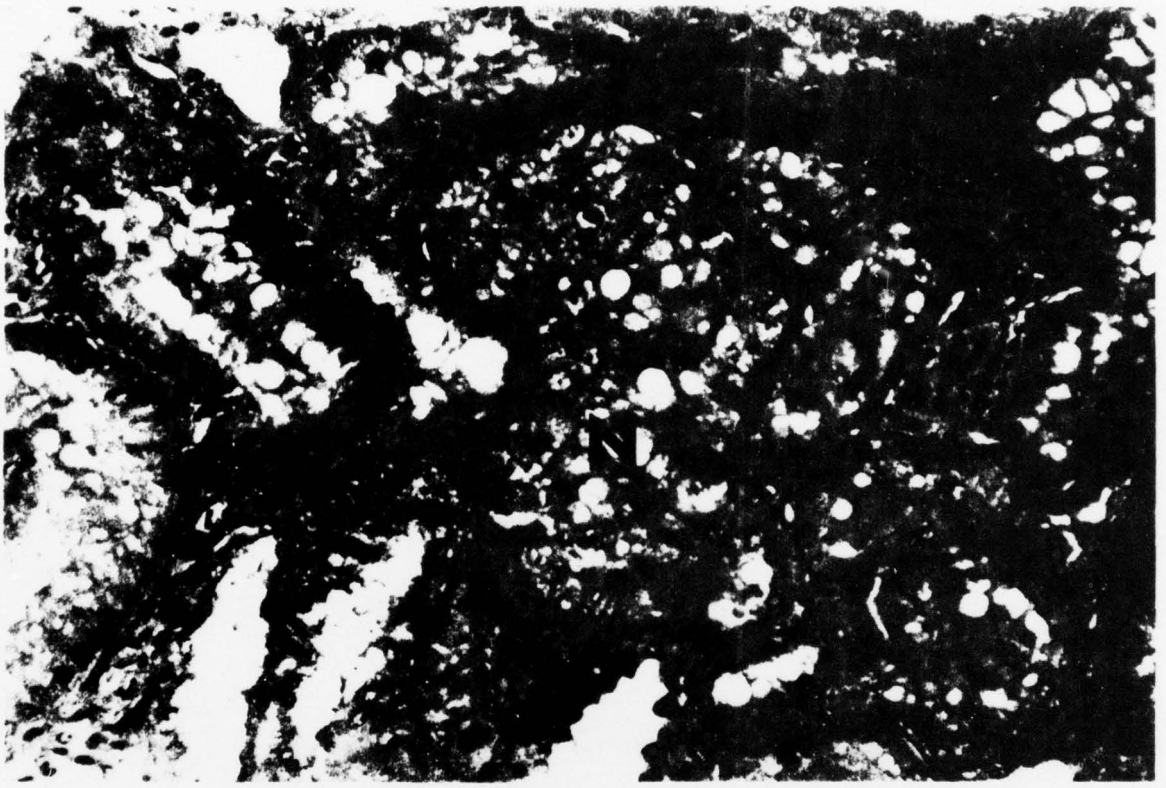

Figure 29 - Tubular necrosis (N) found in an animal sacrificed 26 hours after decompression.

In comparison with normal bone taken from control pigs showing well-defined blood-filled vessels within the Haversian canals (Fig 30), the specimens of cortical bone taken from the two hour pig showed a complete absence of blood in any vessels within these systems, presumably because of air filling (Fig. 31). By four hours and 26 hours after decompression, hyaline thrombi had occluded most of the vessels (Fig. 32).

In this series of animals no thrombi were found in lung tissue. Air-distended pulmonary arteries were found occasionally in the two hour specimen but more prominently and extensively in the four hour animal (Fig. 33).

Electron Microscopy:

Electron microscopic examination of the tissues added new dimensions to our study because it provided ultrastructural observation of the early damage which resulted from this single dive. The bone specimen area examined in Figure 34 does not include the injured vessel from which the hemorrhage occurred; however, the presence of erythrocytes as well as plasma proteins indicates the severity of the damage which allowed blood cells through its wall.

Hemorrhagic areas such as these (seen extensively by light microscopy) were observable in four and 26 hour bone marrow specimens.

The configuration of a normal capillary (Fig 35) surrounded by normal marrow fat cells contrasts sharply with a capillary removed from a pig four hours after decompression showing vessel walls distended by a large air bubble whose interface with blood is characterized by a distinct lipoprotein skin (Fig. 36). Seen in the plasma pushed aside by the bubble is a helmet-shaped red cell which has undergone this transformation in response to stress.

Vessels which were not distended by air were often noted to be contracted as seen in Fig. 37. Degenerating endothelial cells were noted projecting into the lumen of the vessel. Ultramicroscopic studies revealed several other ways in which the endothelium responded to blood-borne bubbles. In some vessels (Fig. 38) the endothelial lining was completely scraped or stripped away while there were even more numerous occurrences of a more simple interruption of the endothelial lining (Fig. 39). In either case, the basement membrane was unprotected.

In Fig. 40, a single platelet is seen adhering of the membrane of a degenerating endothelial cell. Although the capillary in Fig. 41 looks relatively normal in that no vessel injury can be seen at this power, the adherence of a platelet to the vessel wall is an unusual occurrence and strongly indicates vessel trauma.

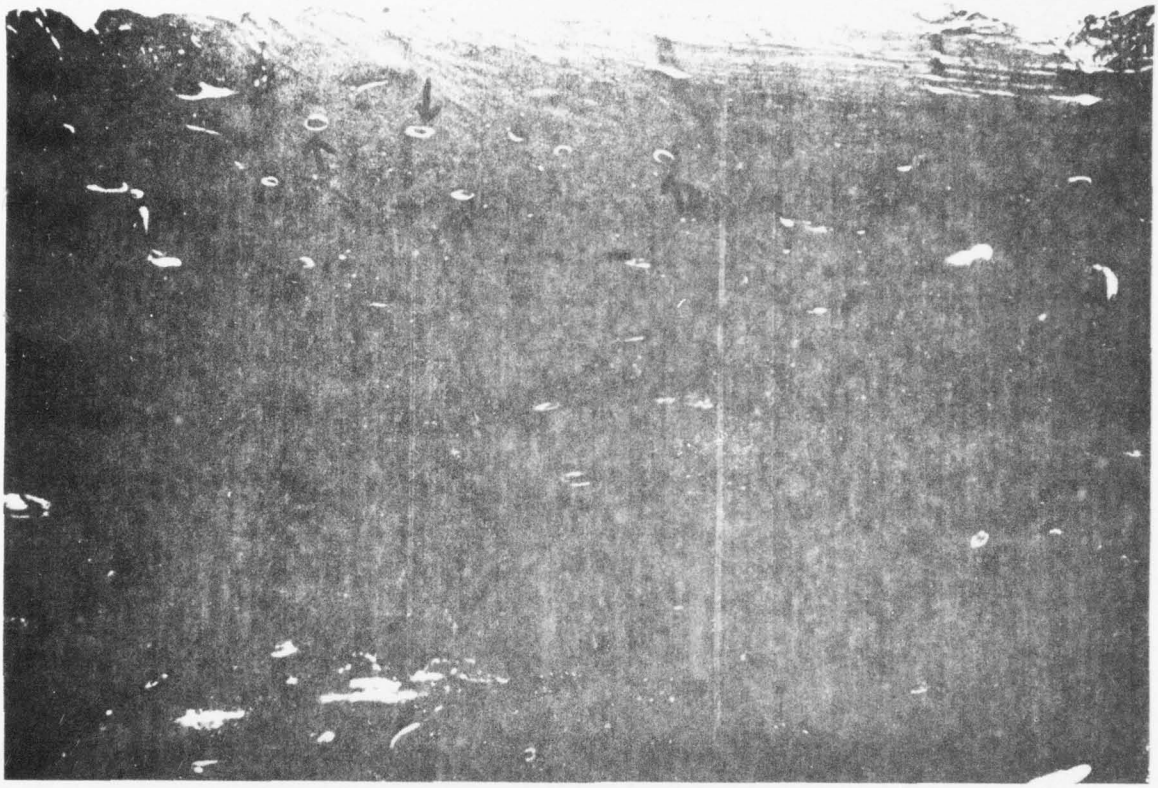

Figure 30- Cortical bone from a normal control animal showing Haversian canals with blood filled vessels (arrows).

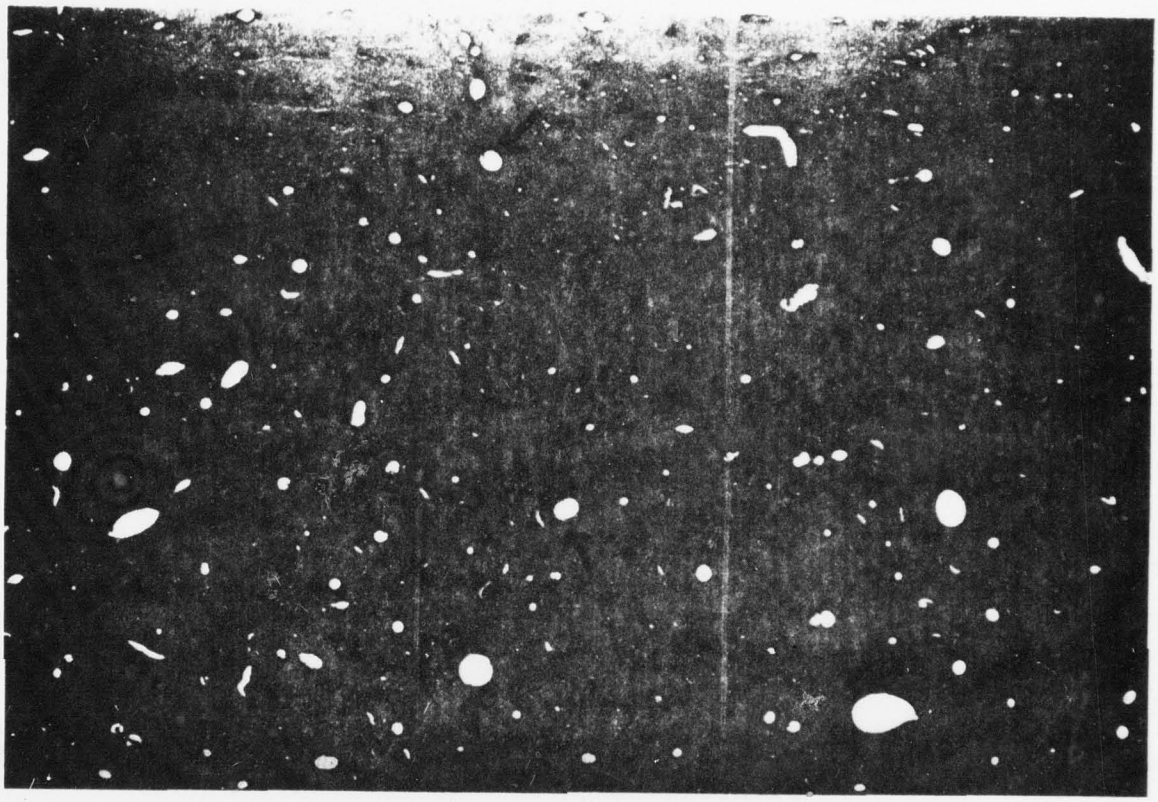

Figure 31 - Cortical bone taken from a pig two hours after decompression showing empty Haversian canals (arrows), presumably because of air filling.

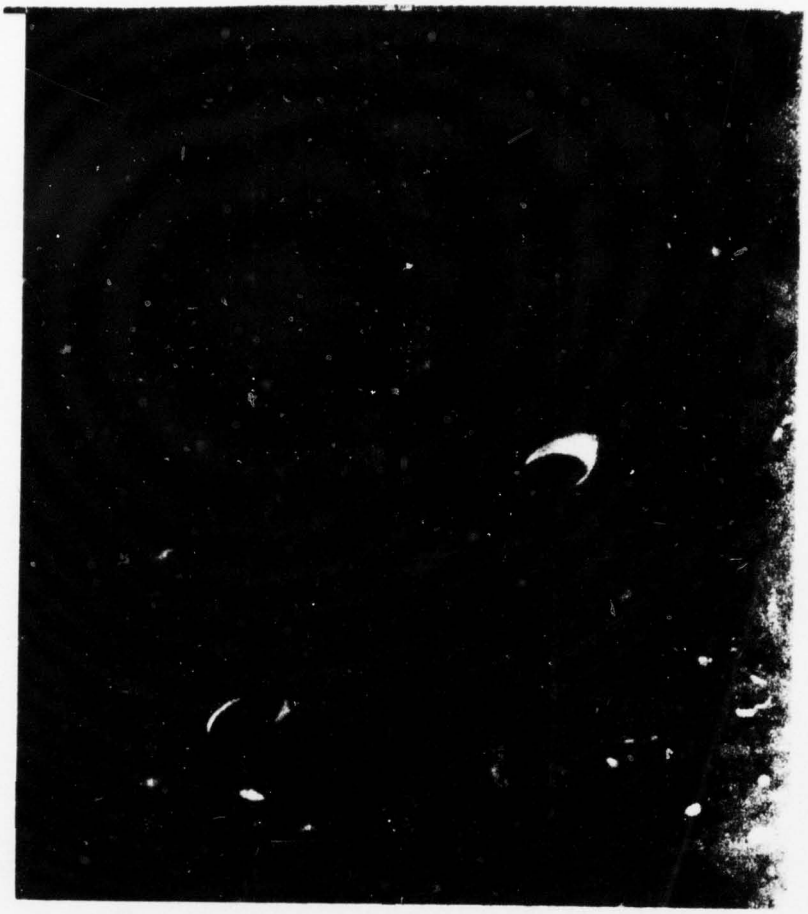

Figure 32- Hyaline thrombi (arrows) occluding vessels in cortical bone by 26 hours after decompression.

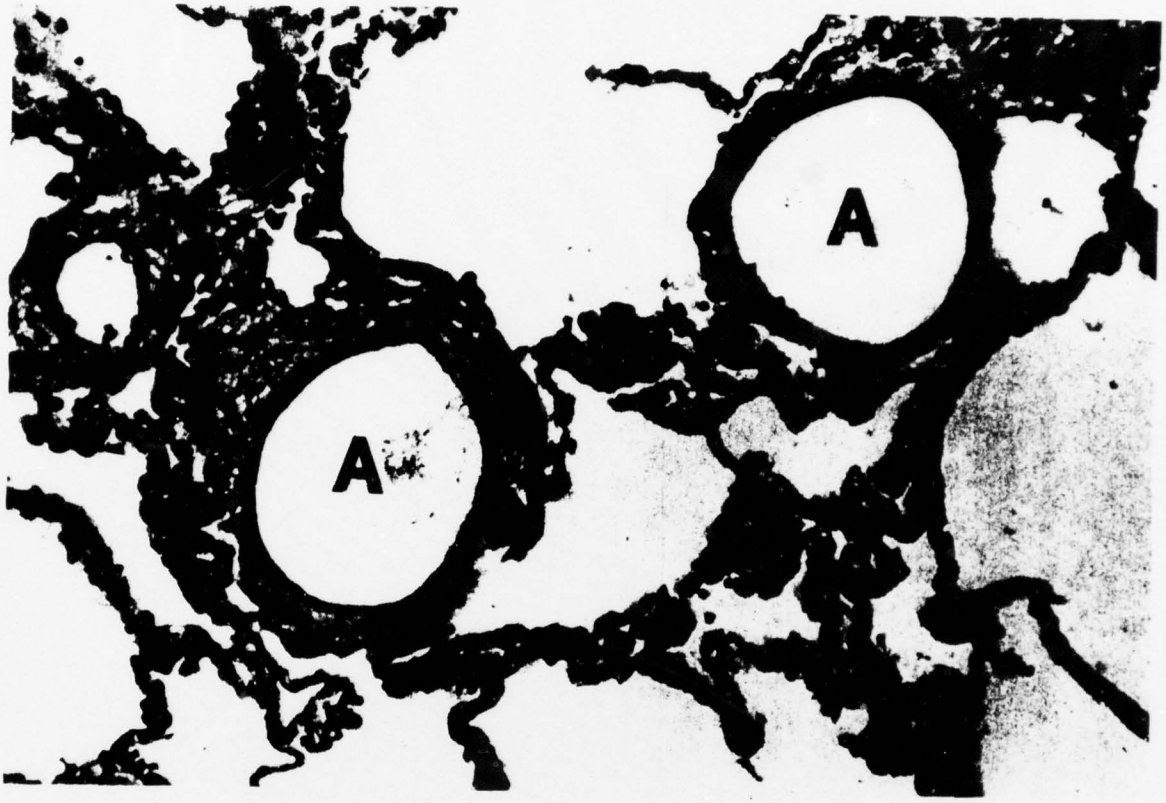


FIGURE 33- Air-distended pulmonary arteries (A) found in a pig four hours after decompression.

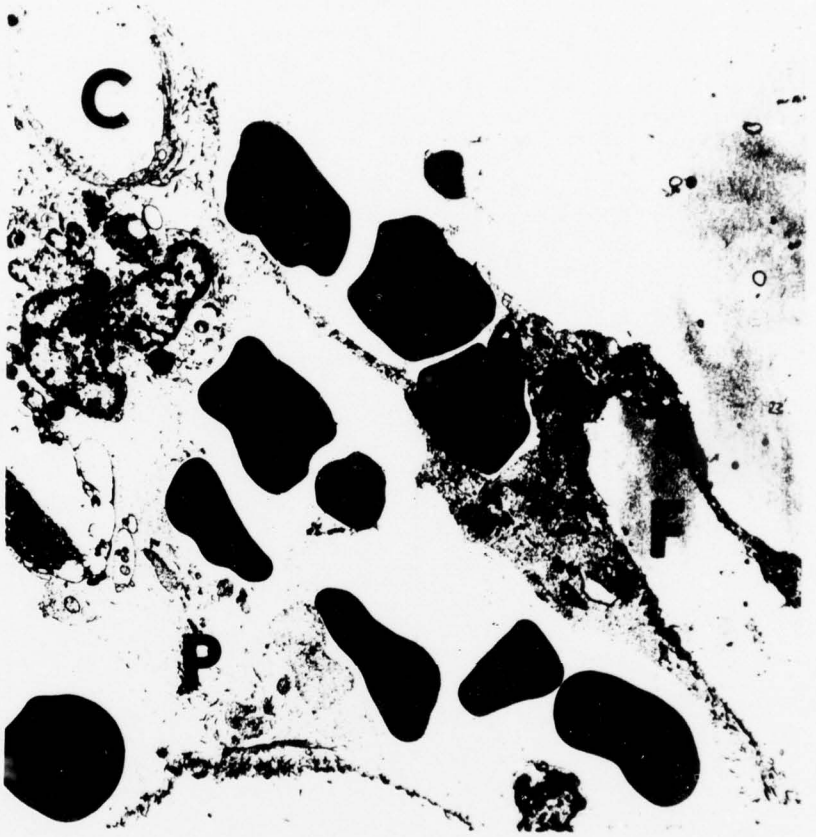


Figure 34. Evidence of vessel damage seen by extravasation of erythrocytes (E) and proteinaceous material (P) into interstitial spaces. A capillary (C) and fat cell (F) are seen in this bone specimen from a pig sacrificed four hours after decompression.

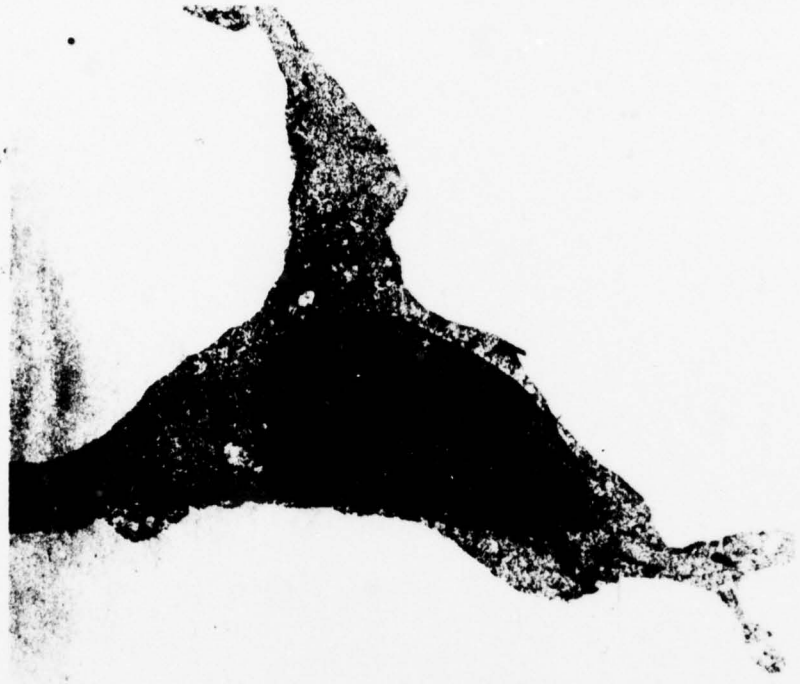

Figure 35 - Configuration of a normal capillary.


Figure 36- Lipoprotein skin (L) marking interface between and air bubble (A) and blood. A helmet-shaped red cell (E) is present in this distended capillary.

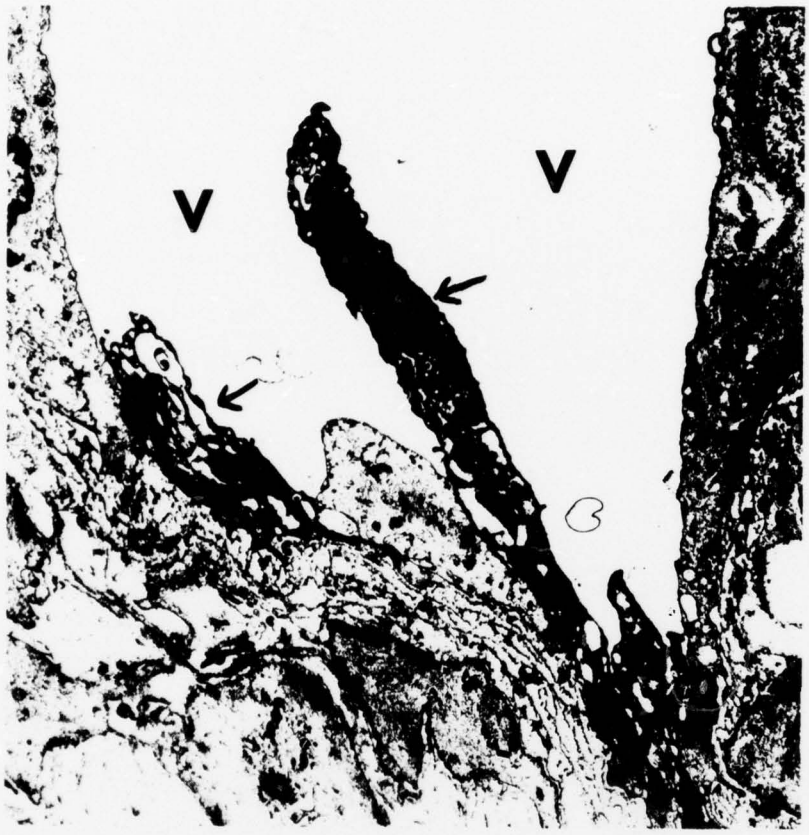


Figure 37. Degenerating endothelial cells (arrows) projecting into lumen of vessel (V).

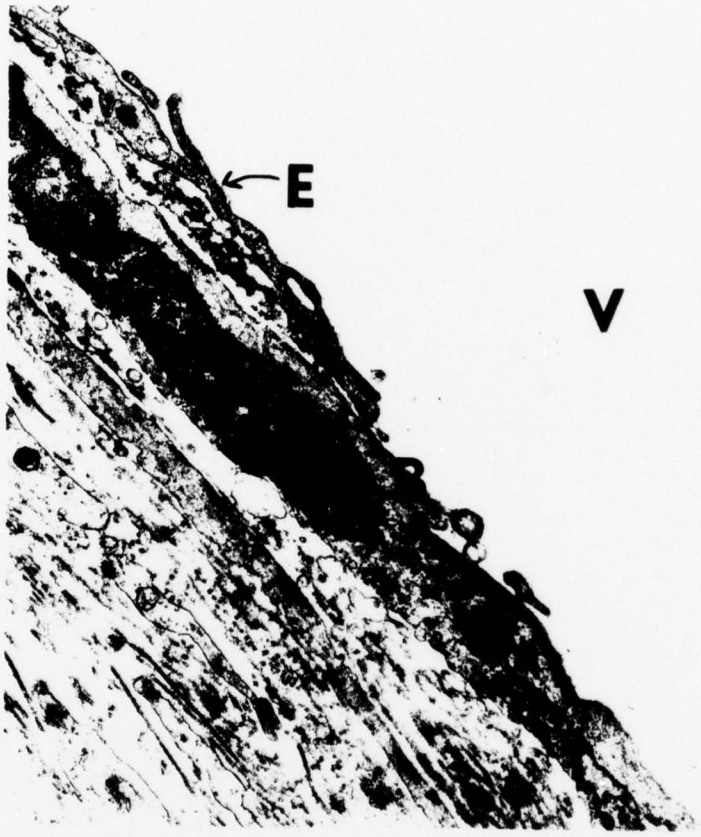


Figure 38 Endothelial lining (E) of lumen (V) stripped or scraped away exposing basal lamina (B).

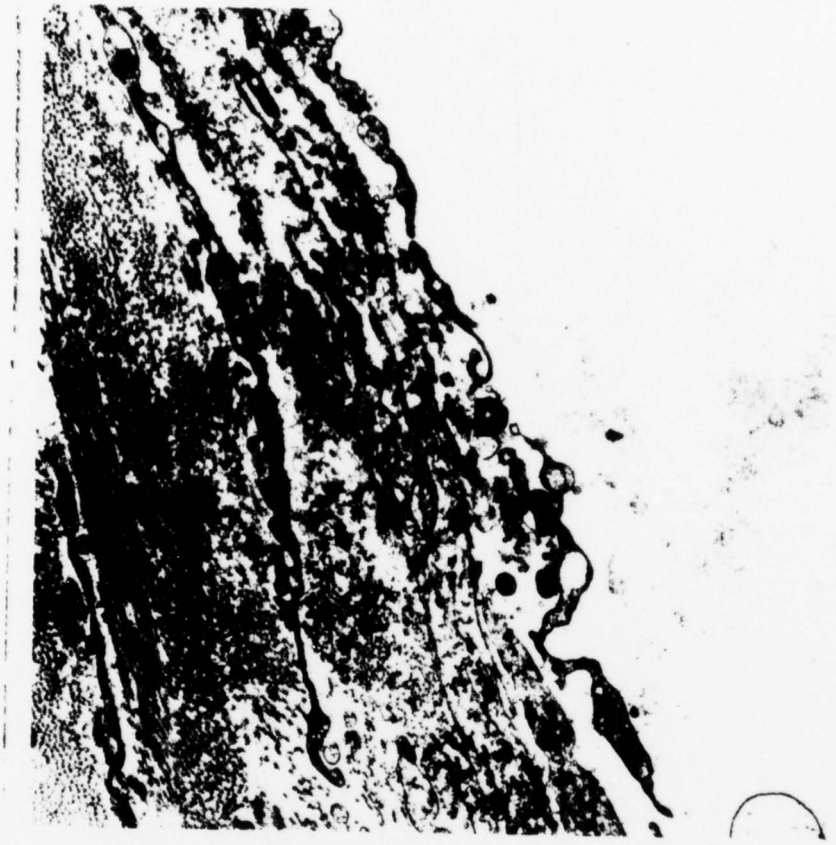

Figure 39 - Cellular debris (D) exposed when endothelial lining was interrupted.


Figure 40 - A single platelet (arrow) adhering to a degenerating endothelial cell (E).

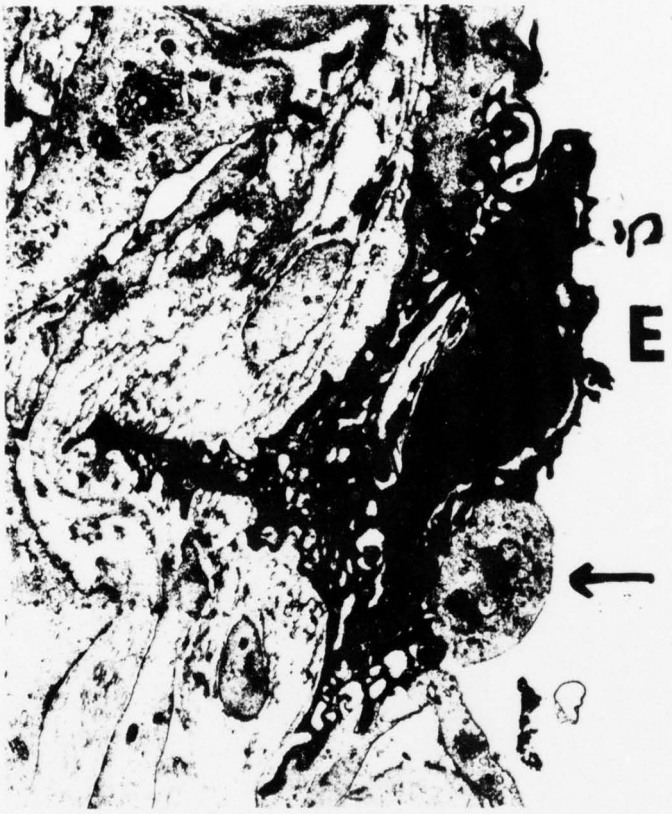


Figure 40 - A single platelet (arrow) adhering to a degenerating endothelial cell (E).

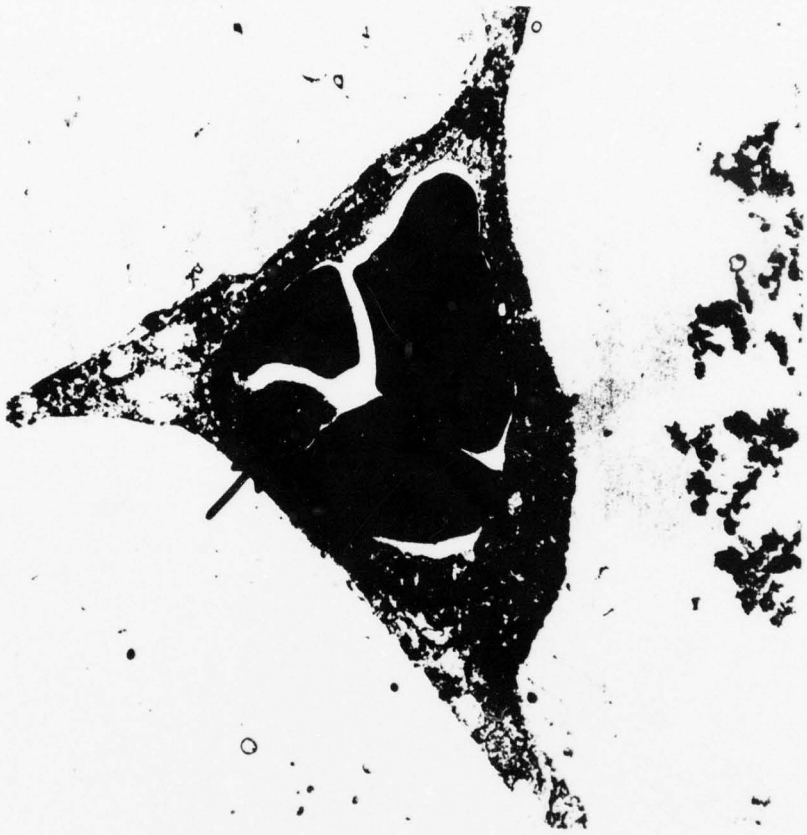

Figure 41- Platelet (arrow) attached to capillary wall.

That severe endothelial damage and microvascular involvement occur following a single inadequate decompression which does not produce signs of decompression sickness is unequivocally documented by this study. That blood-borne bubbles interact with endothelia in various destructive ways is also evident from this study, and confirms the hypothesis of endothelial damage derived from our hematologic studies. That basal lamina is exposed and that platelets are seen adhering to degenerating endothelial cells as well as vessel walls is presented as partial explanation for the consumption of platelets and fibrinogne documented in studies reported previously. That bubbles continue to persist in cortical bone for as long as 26 hours following this single exposure is significant in terms of previous studies which noted that animals held for several months following a single dive later developed extensive osteonecrosis, reinforcing our conclusion that a single exposure may do irreparable harm.

Endothelial damage was observed in several ways: lifting of cells from their basement membrane; sloughing of cells from vessel walls in large areas; degeneration of endothelial cells seen in various phases of injury, from edematous cells to necrotic ones; disjoined and ruptured endothelial cells exposing cellular debris; and projection of endothelia into the lumen of contracted vessels. However the injury manifested itself, it followed that basal lamina was exposed, thereby setting the stage for reactive platelet consumption. Extravasation of vascular contents to the

extent that red cells as well as plasma proteins escaped the vessel lumen indicates the severity of the injury and leads to ischemia, not only by virtue of pressure the presence of additional cellular material and fluids of place on the microvasculature but also because of the thrombus formation within the original traumatized vessel.

The added but unexpected findings of glomerular and tubular necrosis of the kidney underscore the fact that there remain more areas of investigation to explore in defining the consequences of diving.

This selected study designed to elicit information regarding the acute changes in the first day following a single injurious dive more clearly defines the initial course or pathogenesis of osteonecrosis, and yet the study in incomplete.

Table 1 is a graphic summary of the histopathologic observations seen in the examined tissue specimens.

Table 1

| Tissue and Observation | Hours post decompression 1 hr. | 2 hrs. | 4 hrs. | 26 hrs. |
|---|---|---|---|---|
| Kidney | | | | |
| Vascular air | + | ++ | ++++ | - |
| Thrombi | - | +(fsw) | +(few) | ± |
| Tubular Necrosis | - | - | - | + |
| Lung | | | | |
| Vascular air | - | ± | +++ | - |
| Thrombi | - | - | - | - |
| Cortical Bone | | | | |
| Vascular air | +++ | ++++ | ++++ | ++++ |
| Thrombi | + | + | + | + |

- = absent; + = infrequently present; ++ = evident; +++ = very evident; ++++ = overwhelming.

B. Effect of elongated decompression.

In our initial attempts to produce dysbaric osteonecrosis we deliberately chose a profile which would insult some of the "long half-time tissues", without consistently producing overt signs of decompression sickness. We achieved this with the 60 fsw/6 hr,/30 fpm decompression profile. We have also been convinced by our data that decompression was the insult and that the severity of the insult, ie, the rapidity of the decompression, determined the extent and perhaps the time of onset of the lesions.

In our previous studies 100% of the animals repetitively exposed to the above profile, have presented changes, radiographically or histologically, consistent with avascular necrosis. In these rapidly decompressed animals, radiologic evidence

was observed, on the average, by the time the animal had experienced 25 exposures.

To determine the effect of an elongated decompression, 6 pigs were decompressed from 60 fsw/6 hrs. on a profile similar to that described in British Work in Compressed Air Special Regulations (1958) for divers working at 26-28 psig for more than four hours. The animals ascended from 60 feet to 13 feet in 2 minutes where they held for 56 minutes; from 13 feet they were brought to sea level in one minute.

Each animal was exposed to 40 dives on a schedule of four dives per week for 10 consecutive weeks. Two of the six animals exhibited signs of bending, one following its 19th dive and the other its 29th. Both required recompression.

Radiographs were taken bi-weekly for comparison with control films taken prior to the initial dive. All post dive radiographs have been read negative, although one animal presented a suspicious radiopacity in the humerus at the end of the dive series. Upon sacrifice, postmortem radiographs of the suspecious area confirmed the "suspicion", but were not sufficient to warrant labeling it a lesion.

Three animals were sacrificed, including one of the two treated for decompression sickness, the one with the suspicious humeral radiolucency and one other. In the animal with the suspicious humeral radiolucency, gross tissue changes were noted in the femur which had not shown up radiographically. No obvious gross changes were observed in the suspicious humerus.

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# G. THE ETIOLOGY OF EXPERIMENTALLY INDUCED DYSBARIC OSTEONECROSIS

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Presented at the annual meeting of the Undersea Medical Society, Miami Beach, Florida, May 12 and 13, 1976.

While the sequence of events leading to osteonecrosis has been hypothesized by a few investigators, the body of evidence supporting these theories is lean. Our 1972 and 1973 findings of altered hemostatic factors in miniature swine following inadequate decompression (namely, that platelet adhesiveness is increased, that platelet and fibrinogen survival rates were decreased, and that consumption of these factors could be altered only by combined drug therapy) led us to a microscopic examination of tissues taken from singly and multiply dived animals. The investigation (utilizing only light microscopy) produced evidence of hyaline thrombi in the blood vessels of Haversian canals, changes in the medium-sized arteries of bone marrow (specifically, myointimal cell proliferation with subsequent lumen narrowing or occlusion), hemorrhage in the trabecular area of the metaphysis, and finally bone necrosis. But it was clear that the limitations of conventional microscopy, as well as the age of the lesions observed, prevented us from uncovering the process which initiated these events, and only hinted at causal relationships.

In 1975 in San Diego we reported preliminary results of an electron microscopy study observing acute pathologic changes in minature swine within the first 24 hours after 30 fpm decompression from 60 feet of sea water for 6 hours. The animals were sacrificed at 1 hour, 2 hours, 4 hours and 24 hours. These tissues were prepared for electron microscopy by the standard methods, embedded in epon and sectioned by an ultramicrotome.

Examination of the kidney, liver and lungs disclosed the presence of gas-distended vessels extensively in the 1, 2 and 4 hour specimens, as well as the finding of widespread hyaline thrombi at 4 and 24 hours.

(Slide 1) Vessel trauma was demonstrated by platelets adhering to degenerating endothelial cells, and (Slide 2) by sloughing of the endothelial lining, thereby exposing the basement membrane to blood flow and triggering events which led to coagulation. Single and coalesced bubbles were found in significant numbers in all animals.

Because of the small number of animals used in this pilot study, four in all, the investigation was expanded to include pigs sacrificed at 8 and 16 hours, as well as an increased number at 2,4 and 24 hours. This report presented today then covers the findings in 16 animals: 1 at 1 hour, and 3 each at 2, 4, 8, 16, and 24 hours, and deals solely with ultrastructural examination of humeral and femoral tissue taken from these animals and compared with control animals. None of these animals exhibited any sign of decompression sickness at the time of sacrifice.

(Slide 3) The aftermath of vessel injury sustained in this single dive is clearly seen by the extravasation of vessel contents, an indicator of vessel trauma. The interstitial area viewed here contains erythrocytes and proteinaceous material. A distended but intact capillary is seen in the field. (Slide 4) The discontinuity of the endothelial lining is evident and (Slide 5) is the precursor to the platelet adhesion seen here.

This most recent study concentrated on cortical bone and contributed evidence that bubbles are present not only in the medullary cavity but also are seen throughout the vessels of the Haversian system and occupy the lacunae of bone cells or osteocytes in cortical bone.

(Slide 6) The configuration of a normal Haversian canal is seen here. A well-filled intact blood vessel is present, with normal endothelial structure; the nucleus of a periocyte and the nucleus of a fibroblast of connective tissue cell are normally distributed within the canal. (Slide 7) A higher magnification of another Haversian system brings the blood vessel closer, and (Slide 8) even closer. Particularly note here the undulating surface of the cytoplasm of the vessel lining which we will come back to later.

(Slide 9) In contrast, a bubble is noted in this Haversian canal, filling the blood vessel and compressing the canal contents. That this is a blood vessel is confirmed by the presence of an endothelial nucleus. (Slide 10) Although the cause is not known, vessel collapse seen in this slide was not a rare finding. The basement membrane can be defined, as well as the endothelial lining. However, the lumen is not patent, and traces of cullular debris are evident within the vessel. We consider that vessel spasm may be in the chain of events which occurs following decompression at this rate.

(Slide 11) A normal osteocyte is noted with normally distributed cellular contents within the lacuna. (Slide 12) However, numerous occurrences of bubbles compressing osteocytic contents

were observed, as seen here, and again here (Slide 13), and again here (Slide 14).

(Slide 15) The normal appearance of cytoplasmic undulation referred to earlier is demonstrated again here compared with the taut appearance of attentuated cytoplasm (Slide 16) which is the result of the gas bubble exerting pressure on it. The striations here are due to the shattering of the epon by the microtome knife. (Slide 17) In some cases, cytoplasm is so stretched that the tension causes a break in the tissue; and then because of diminished cellular integrity, cytoplasm is lost from the vessel surface. This finding correlates well with our earlier hypothesis of vessel trauma suggested by changes in platelet and fibrinogen survival rates and platelet adhesiveness.

(Slide 18) A bubble is again seen in this section of a Haversian canal. A blood vessel is totally dilated with a gas bubble, its endothelial surface smooth without undulation. An endothelial cell nucleus can be seen at its edge. (Slide 19) The difference between the appearance of a gas bubble and an artifact is demonstrated here. The break or tear in the tissue probably occurring during slide preparation is contrasted with a space occupying gas bubble now filled with epon. Again the striations indicating epon shattering are present.

(Slide 20) Although large single bubbles were more the rule, coalesced bubbles such as those seen here in a Haversian system were an occasional finding.

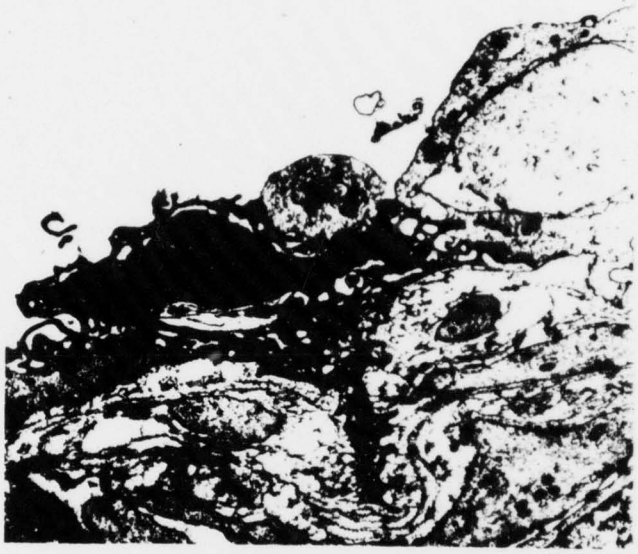
The results of this inquiry, coupled with the findings of the 1975 investigation, confirmed the presence of injurious gas bubbles in the vasculature of cortical bone. The additional

discovery of gas within the lacunae of individual bone cells or osteocytes is further persuasion that gas bubbles initiate a continuum which culminates in bone necrosis.

We suggest therefore that the etiology of experimental dysbaric osteonecrosis is gas, and the pathogenesis of the disease is bubble-induced endothelial damage with subsequent thrombus formation, vessel wall thickening leading to lumen narrowing with reduced blood flow or vessel occlusion, extravasation of vessel contents causing extravascular pressure in interstitial spaces, and gas-filled lacunae with displacement of osteocytic contents.

This work was supported by the Office of Naval Research Contract N00014-71-C-0273 with funds provided by the Naval Medical Research and Development Command.

Slide 1



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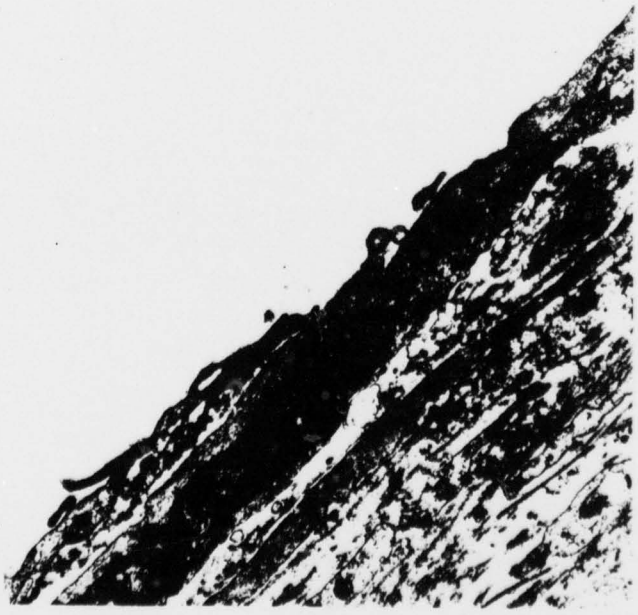

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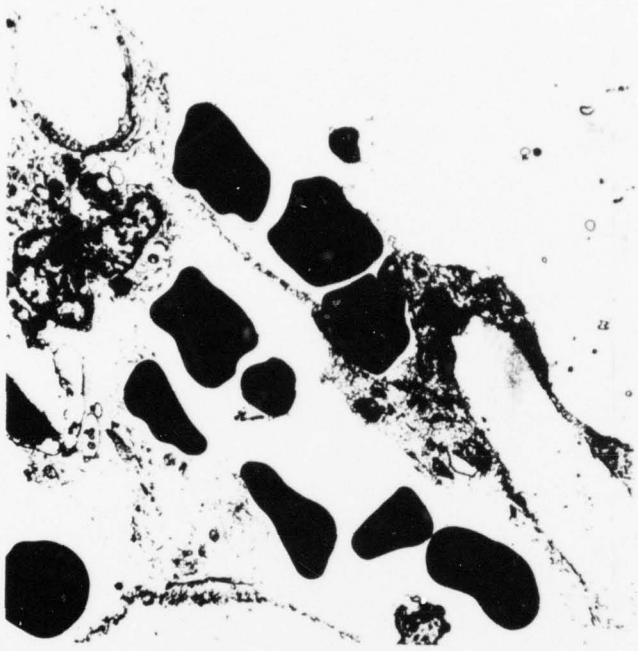

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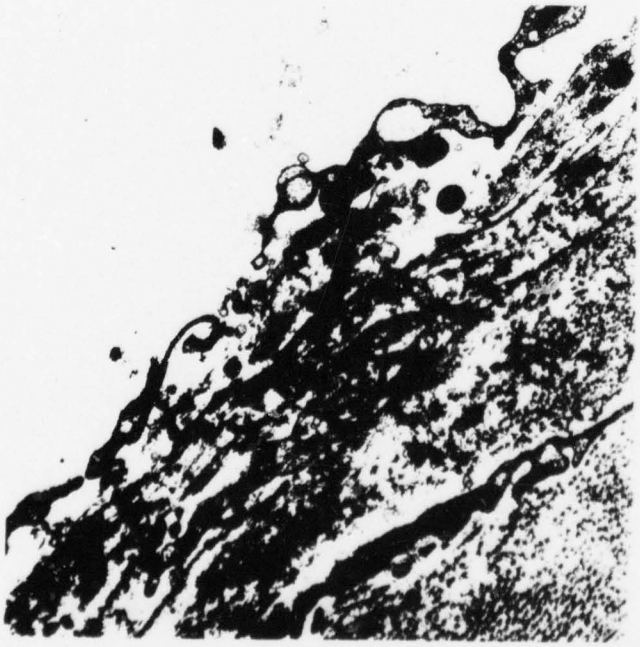

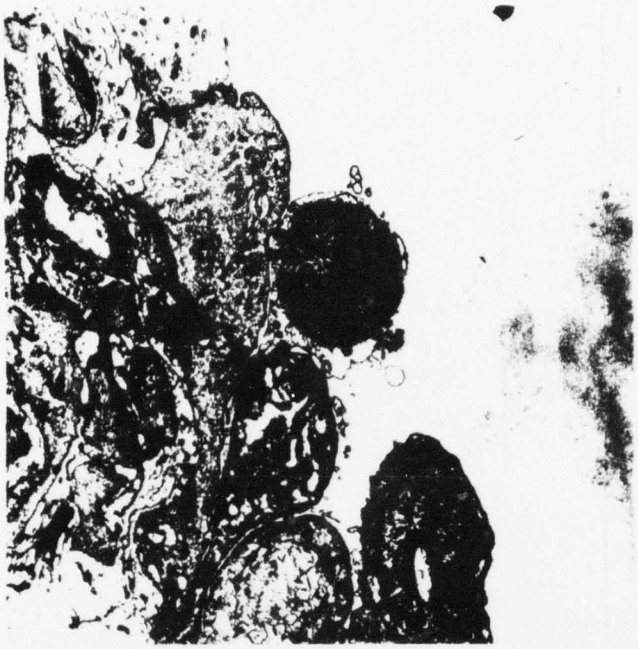

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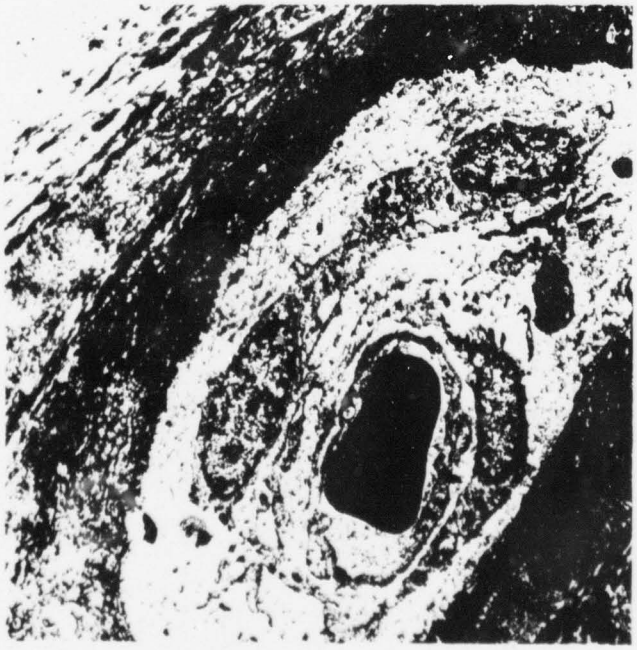

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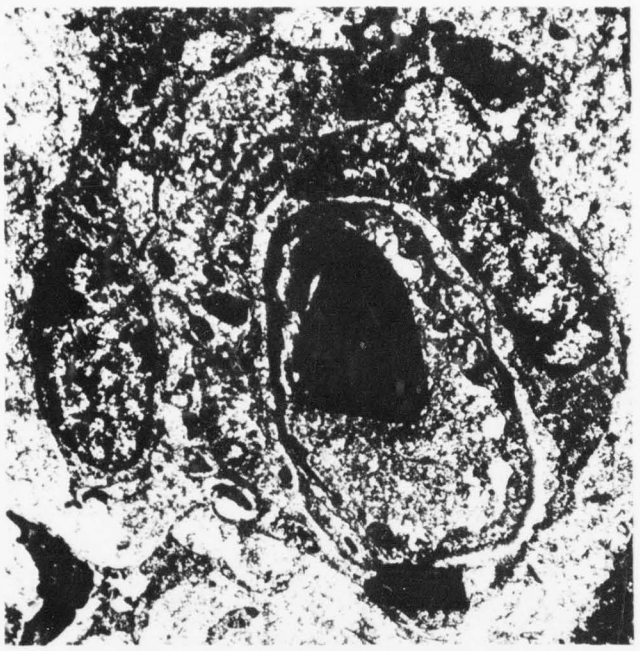

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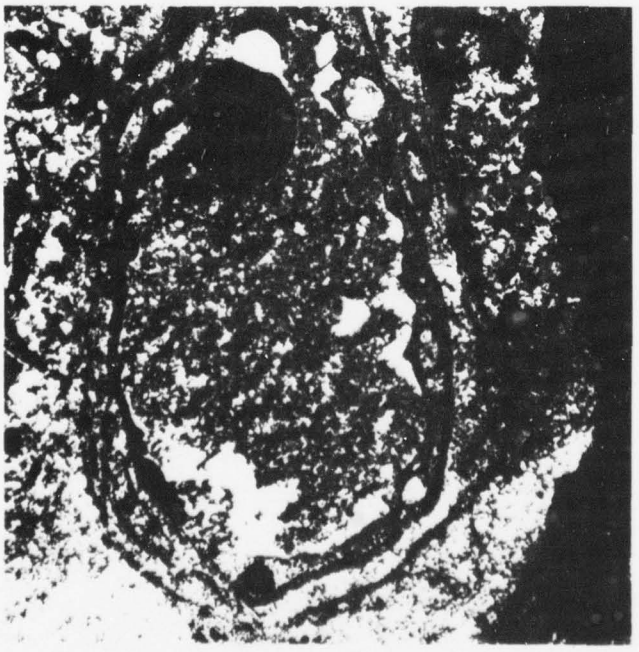

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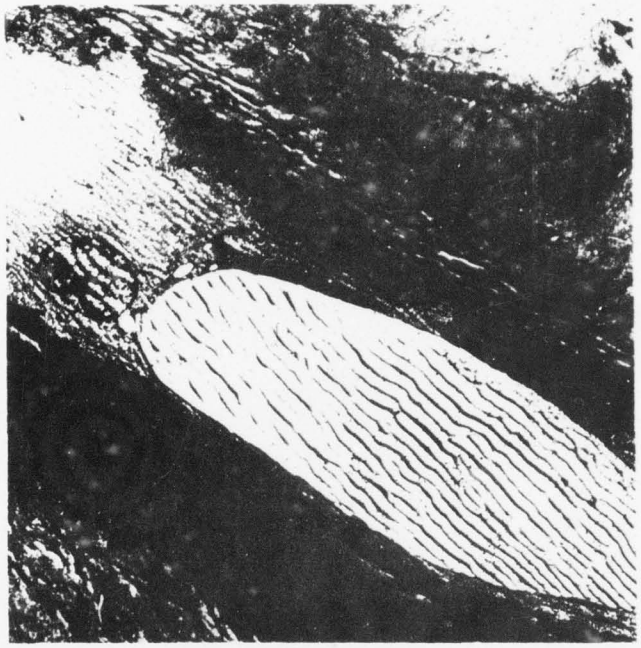

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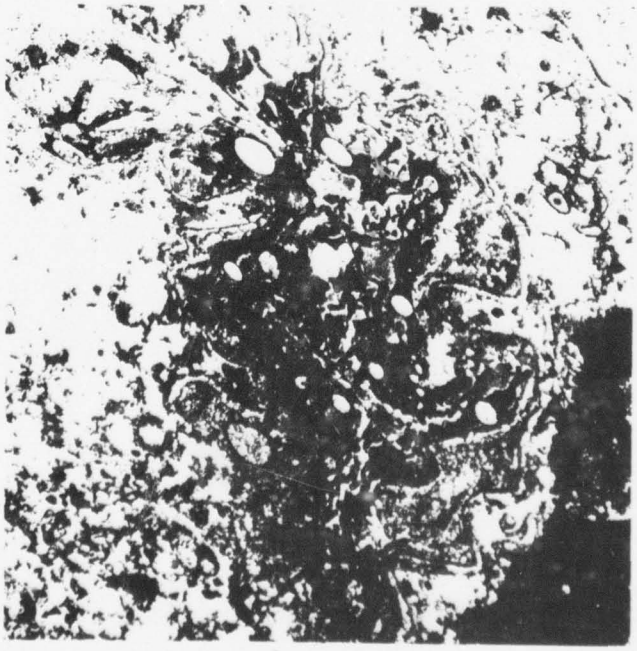

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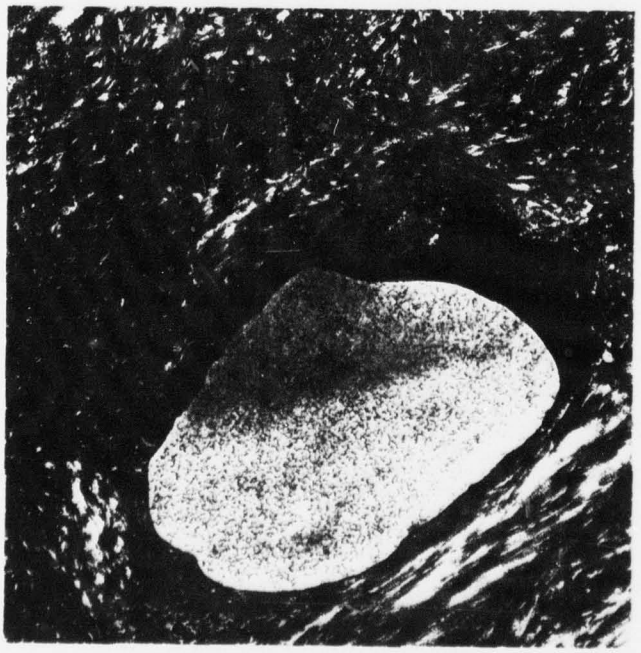

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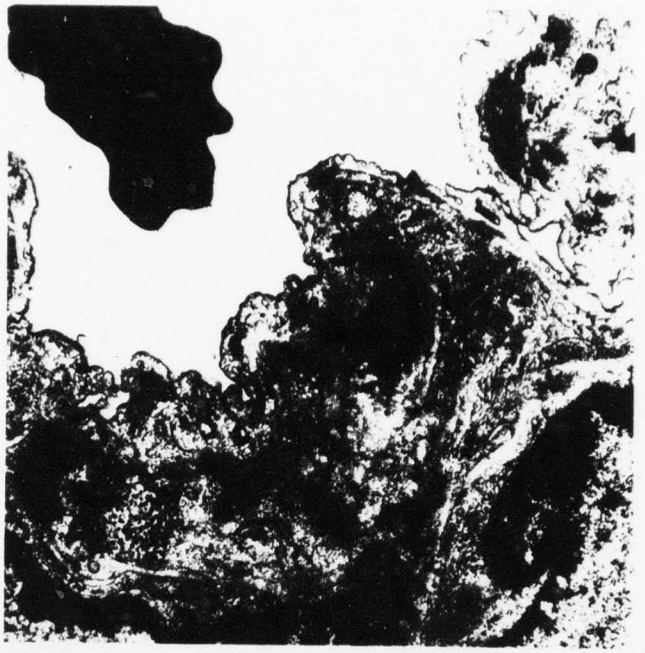

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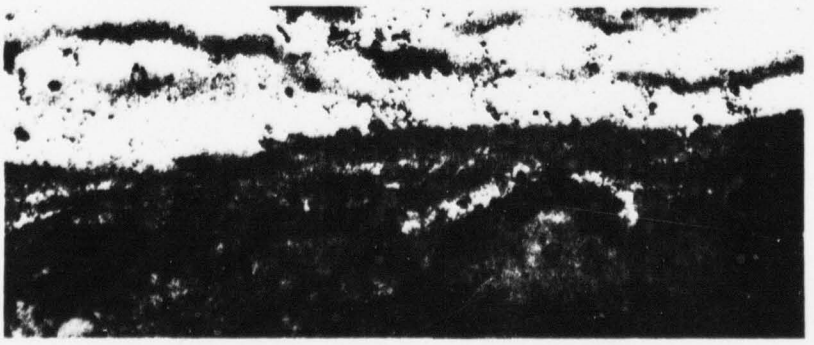

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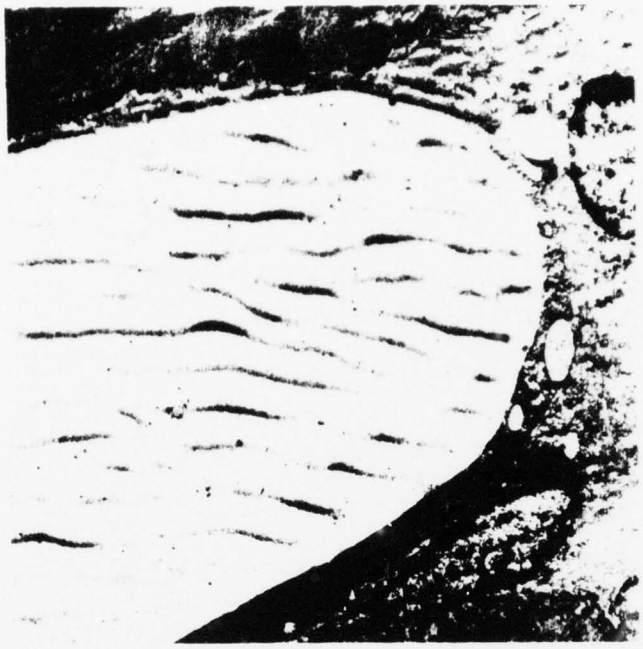

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IV. Collagen Investigation

Although collagen has long been recognized as a major organic constituent of bone, extreme insolubility has deterred understanding of this highly crosslinked material. The intimate involvement of collagen in calcification of hard tissues such as bone and teeth has been clear for some time (47) although the mechanism of calcification still defies explanation (2 ).

The unique composition of collagen has provided an amino acid, hydroxyproline, with which to follow some aspects of anabolism or catabolism of this connective tissue protein. However, the metabolism of bone collagen is affected by hormonal control (10; 31) and vitamin influences (3 ; 11). It would be difficult to assess the catabolism of bone by measurement of urinary excretion of hydroxyproline (62). Other means of determining involvement of bone collagen in osseous tissue modifications must therefore be sought.

Since collagen is a major constituent of the organic matrix in bone, it is probably affected in either a primary or secondary manner in osteonecrosis. Approximately 20% of the total dry weight of bone is collagen. The total content of collagen has been determined from the hydroxyproline values and by extraction as gelatin (13). Of the total organic material in the bone, collagen comprises from 93-95% of this organic matrix, the remainder being mucopolysaccharide or lipid in nature (13). This collagen would be predominantly from the osteoid tissue but would include vascular collagen

as well, especially in the diaphyseal (shaft) region.

Collagen of bone has been studied in various species including the chick (33), swine (5 ), and the growing rat (12;8 ). The studies have been often in the presence of a lathrogen β-aminoproprionitrile which inhibits the cross-linking of collagen. Introduction of a crosslinking inhibitor such as β-aminoproprionitrile into either an in vivo or in vitro system increases the solubility of newly formed collagen to a certain extent. However, most bone collagen remains insoluble and can be dissolved only in the order of 0.5% to 1.0% with 0.5 M acetic acid, which at the same time decalcifies the tissue, presumably then releasing more collagen to be dissolved. The completion of decalcification results in about 99% of the collagen remaining insoluble. Reagents which have a higher chemical activity must be utilized and result in denaturing and gelation of the collagen. Therefore, re-extraction of the residue of bone remaining following acetic acid extraction by reagents such as urea, guinidine HCl, lithium chloride and potassium thiocyanate have been used (12 ;18 ;33 ) to yield more of the total collagen in a soluble form.

The yield of uncrosslinked α chains and of crosslinked β and γ chains indicates the degree of maturation of the collagen extracted in most cases. Quantitative determinations may be made with acrylamide gel electrophoresis followed by densitometer scanning of the protein bands separated on the gel. Other chemical modifications which occur in collagen

fractions can be studied in order to gain understanding of changes associated with normal and possibly altered properties of the bone collagen. These include the quantity of aldehyde groups on the collagen chains. The number of aldehydyes derived from lysine residues is an indication of the ability of the chains to eventually crosslink. The presence of aldehydes may therefore be indicative of the remodeling ability of the collagen or of the lability of crosslinks once they are formed. This is to say, a high number of aldehydes does not necessarily indicate a high degree of crosslinks and therefore insolubility; it may only indicate the attempt to form crosslinks or that crosslinks once formed were not reduced in such a manner as to create stability.

Therefore, we have made both quantitative and qualitative determinations of the bone collagen from the femur and humerus in the proximal epiphyseal, metaphyseal and diaphyseal regions of miniature swine. These determinations consisted of the (1) total collagen, (2) collagen extractable using solutions of salts of increasing chemical activity, (3) quantity of monomer ($\alpha$) chains compared with dimer ($\beta$) chains and trimer ($\gamma$) chains, (4) quantity of aldehydes in all the different extracted collagens, (5) total amino acid composition of the collagens from each region and from each type of extraction.

By examination of the biochemical characteristics of bone collagen from animals with diagnosed aseptic bone necrosis and from control animals we would detect differences in short

term involvement of bone collagen and be able to indicate a direct or indirect contribution of collagen metabolism to the observed bone changes.

A. Methods

Source of Sample: Bones removed from miniature pigs were divided into two groups, control and necrotic, according to the radiographic diagnosis. Bone slices were made longitudinally and frozen until analyzed. The bone slices were washed in cold saline to remove particles resulting from sawing, and freed of muscle, periosteum and tendon, using a scalpel. The marrow was rubbed away with fingers to avoid damage to the bone in those regions. Articular cartilage was removed to a depth of 2 mm below the cartilage.

Preparation of Samples for Extraction: Longitudinal bone slices from the upper part of the femur and humerus were divided into three regions: (1) epiphyseal ring, 2 mm under the cartilage and approximately 0.5 cm below the ring; (2) metaphysis, below the epiphyseal ring area and above the diaphysis; and (3) the diaphysis, approximately 2 to 3 cm in length. The areas were separated using a hammer and wood chisel. The bone pieces were defatted in one volume of water at 4° with chloroform:methanol (2:1). Following several water washes the pieces were freeze dried. The defatted bone sections were reduced to smaller pieces with a hammer and subsequently ground by hand in a heavy mortar and pestle. All of the samples were pulverized until the

particles would pass through an 80 mesh (USA) sieve. All subsequent procedures were carried out at 4°.

Collagen Extraction Procedures: Sequential extractions, using five different solutions, were made on 34 bone samples from 8 animals. All extractions were for a minimum of 3 changes of extracting solution and some extractions required 20 days for completion. The supernatant solution containing the soluble collagen was centrifuged at 16,000 x g for 30 minutes. The liquid phase was carefully pipetted off the residue and retained until the total number of extractions was completed. The extractions were discontinued when the ultraviolet absorbance at 230 nm was reduced to one-tenth that of the first extraction solution; this was usually after 3 times. In each case, the bone residues were washed with water 3 times to remove the solvent solution. The first wash was pooled with the extraction.

The solubilization procedure of collagen from the finely ground bone samples was carried out in several different ways. The first removal of collagen in 0.5 M acetic acid, 0.01% Triton-X 100 also served to substantially decalcify the bone. This extraction was best carried out in dialysis bags. Inorganic components which would pass through dialysis tubing could then be removed at the same time collagen was being solubilized and retained inside the dialysis bag. Completion of calcium removal was determined between each of the acid extractions by means of a precipitation test.

Demineralization of bone: Dialyzer-Rocker Shaker apparatus (Fig. 27) used for the acetic acid extraction step was designed in our laboratory for that purpose. Briefly, it consists of heavy wall glass tubing, I.D. 2.4 cm and length 24 cm. Dialysis bags were tied securely at one end leaving an extension of dialysis tubing approximately 4 cm long. The bone sample, 1 gm, 5 ml acetic acid solution and 4 glass beads, 3 mm diameter, were placed in the dialysis bag. Residual air was removed and the bag tied securely to give a length of 22 cm which would fit inside the glass tubing. One extension of the dialysis tubing was brought out through the smooth end of the glass tubing and secured in place with a rubber stopper. The second extension of dialysis tubing was brought out of the opposite end and the glass tubing filled with the same solution being used for extraction. The second rubber stopper was then placed securely in the glass cylinder and checked for leaks. Up to 10 such tubes could be placed on each side of a rocking platform. The platform made approximately 30 excursions per minute with a displacement of 10 cm at the outer edge. The dialyzate was changed daily for 14 days.

Extractions with the remaining solvents were performed in 17 ml plastic stoppered tubes with 4 glass beads to facilitate mixing. Samples were again placed on the rocker platform shaker. Supernatant solutions containing soluble collagen were recovered by centrifugation at 16,000 x g for 30 minutes. Pooled supernatant solutions were dialyzed against 0.15 M acetic acid to remove small molecules and the

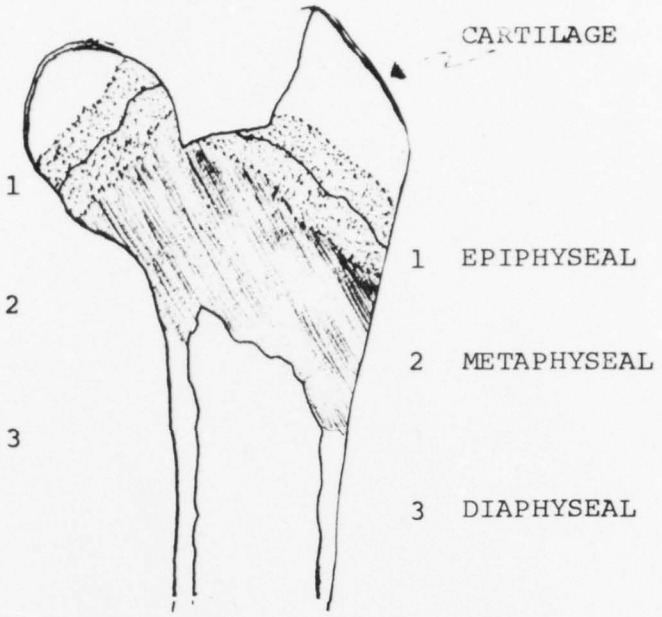


Figure 26 Method of sampling proximal femur, showing regions, F-1,F-2, and F-3. A similar sampling was taken for the humerus.

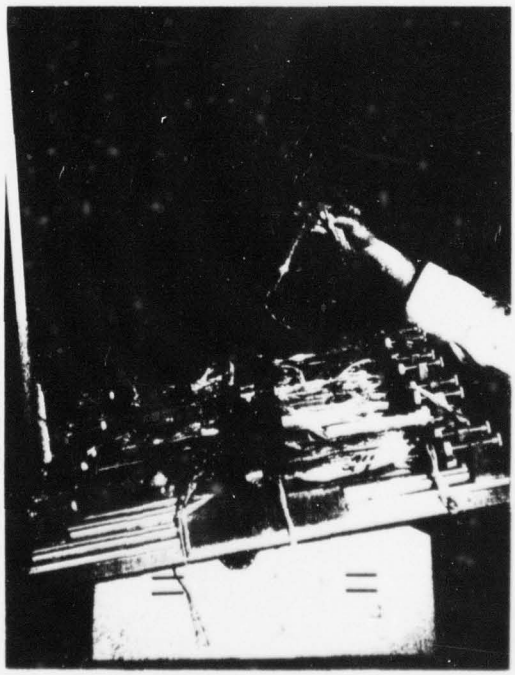

Figure 27 Rocker-shaker apparatus showing tubes containing dialysis bags.

extracting solvent. This dialysis procedure sometimes required up to 3 weeks for complete removal of all salts. The resultant salt-free extracts were shell-frozen and freeze-dried for subsequent weight determination.

The acetic acid soluble collagen was subsequently purified and separated into 2 fractions by precipitation with 5% and 20% sodium chloride using previously defined procedures (41). The collagen was redissolved in 0.5 M acetic acid, dialyzed salt-free and freeze-dried.

Total Amino Acid Analysis: Collagen samples of 1 mg were hydrolyzed in 6 N hydrochloric acid under $N_2$ in vacuum at 108° for 20 to 44 hours. Hydrochloric acid was removed with an Evapo-Mix (Buchler). Total amino acid content was determined on a Beckman 120-B Amino Acid Analyzer.*

Hydroxyproline and Proline Determinations: The 2 imino acids were released by hydrolysis, separated on columns of Dowex 50 x 8 (200-400 mesh) and determined quantitatively as described by Richmond and Stokstad (46).

Aldehyde Estimations: Approximately 4 mg of collagen were digested with bacterial collagenase (26). Aldehydes in the supernatant solution were were determined using colorimetric (38) procedure as standardized with acetaldehyde.

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*We are indebted to Dr. W. S. Rickert, Dept. of Statistics, University of Waterloo, Waterloo, Ontario, Canada, for most of the amino acid analyses.

Acrylamide Gel Electrophoresis: The procedure of Goldberg, et al. (19), using 5% acrylamide was adapted for use in the slab gel cell of ORTEC. Samples of 10 and 25 µg were compared with standard guinea pig skin neutral salt soluble collagen. The protein bands were stained with Coomassie Blue and densitometer tracings were made of photograph negatives.

B. Results

Collagen in the head and upper shaft of the long bones of miniature swine has been studied in animals subjected to various regimens of a diving protocol designed to produce aseptic bone necrosis. Following identification of bone lesions by procedures described elsewhere in this report, the long bones were divided into regions as shown in Figure 26.

The reduction of bone particle size to a fine powder was mandatory in achieving consistent and complete extraction of the collagen matrix, especially with 0.5 M acetic acid (data not shown and as determined from literature reports). Therefore, the bones were ground to a mesh of 80 (USA standard) to facilitate reproducible collagen solubility. Homogenization equipment was not available at the time for grinding the tissue after the decalcification step.

The total quantity of collagen in bone tissue from different animals such as growing rats ( 8;12) and in bones from other sources such as rabbit, ox and human (13) and swine ( 5) has been analyzed in other laboratories. Values for collagen are clustered around 20% of the dry weight of

bone and were estimated from hydroxyproline content as well as from total amino acid analysis of autoclave and salt-extracted collagen.

Our report verifies (Table 5) the approximate value of 20% collagen per total dry weight and, by analysis of well-defined areas of the femur and humerus, extends the present knowledge of collagen content. The bone of the upper femur, epiphyseal region F-1, well below the cartilage area, had an average collagen content of 21.61% for animals with diagnosed aseptic bone necrosis, while the same area from control animals had slightly less collagen, 20.9%.

Interestingly, the lowest average collagen content of the femur regions was in the metaphysis F-2 area, where the experimental animals had 18.02% and the control 16.98% of the total dry weight. The area of the femur toward the shaft had similar collagen content of 19.08 and 19.00% for both experimental and control animals. Collagen content for the predominantly shaft region was intermediate between the 2 areas.

It should be pointed out that collagen content determined for the bone regions represented collagen from all sources: calcified tissue and vascular system. Collagen accounts for almost all of the extractable component, while elastin, if present, would be in the insoluble residue.

The humerus of the experimental animals presented a somewhat different relationship compared to the femurs from the same animals. The highest collagen content rather than the lowest was found in the H-2 metaphyseal area, with an

Table 5

TOTAL CONTENT OF PROLINE AND HYDROXYPROLINE IN BONE SAMPLES

Bones were from animals exhibiting necrosis (see text for diagnostic procedures) and from controls and represent the three different areas studied of the femur and humerus. Values are given as µMoles amino acid per 100 mg defatted, pulverized dry bone.

| Bone Area Designation | Animal No. and Treatment | µMoles/100 mg Bone Hydroxyproline | Proline | Proline / Collagen Hydroxyproline | mg |
|---|---|---|---|---|---|
| Femur - $F_1$ | Exp. 204 | 18.95 | 23.94 | 1.263 | 20.28 |
| " | " 117 | 19.90 | 24.81 | 1.247 | 21.30 |
| " | " 104 | 21.75 | 23.30 | 1.071 | 23.27 |
| " | " Average | 20.20 | 24.02 | 1.194 | 21.61 |
| " | Control 124 | 19.53 | 23.65 | 1.211 | 20.90 |
| Femur - $F_2$ | Exp. 204 | 17.07 | 18.32 | 1.073 | 18.26 |
| " | " 117 | 17.00 | 19.83 | 1.153 | 18.19 |
| " | " 104 | 16.26 | 17.91 | 1.101 | 17.40 |
| " | " Average | 16.84 | 18.67 | 1.109 | 18.02 |
| " | Control 124 | 15.87 | 20.72 | 1.306 | 16.98 |
| Femur -$F_3$ | Exp. 204 | 19.04 | 21.71 | 1.140 | 20.31 |
| " | " 117 | 17.17 | 18.00 | 1.048 | 18.37 |
| " | " 104 | 17.29 | 17.17 | 0.993 | 18.50 |
| " | " Average | 17.83 | 18.96 | 1.060 | 19.08 |
| " | Control 124 | 17.76 | 18.96 | 1.060 | 19.00 |
| Humerus - $H_1$ | Exp. 106 | 22.17 | 27.06 | 1.220 | 23.72 |
| " | " 117 | 21.31 | 23.58 | 1.106 | 22.80 |
| " | " 104 | 18.78 | 21.88 | 1.165 | 20.09 |
| " | " 121 | 17.48 | 18.68 | 1.067 | 18.70 |
| " | " Average | 19.94 | 22.80 | 1.140 | 21.34 |
| Humerus - $H_2$ | Exp. 106 | 20.15 | 24.88 | 1.235 | 21.56 |
| " | " 117 | 28.00 | 25.62 | 0.915 | 29.96 |
| " | " 104 | 20.64 | 22.96 | 1.112 | 22.08 |
| " | " 121 | 22.06 | 22.98 | 1.042 | 23.60 |
| " | " Average | 22.71 | 24.11 | 1.076 | 24.30 |

| Bone Area Designation | Animal No. and Treatment | µMoles/100 mg Bone Hydroxyproline | Proline | Proline/Collagen Hydroxyproline | mg |
|---|---|---|---|---|---|
| Humerus - $H_3$ | Exp. 106 | 15.96 | 17.21 | 1.082 | 17.08 |
| " | " 117 | 20.32 | 19.28 | 0.949 | 21.74 |
| " | " 104 | 19.53 | 18.15 | 0.929 | 20.90 |
| " | " Average | 18.58 | 18.21 | 0.987 | 19.88 |

average of 24.3% (Table 5). Less collagen was in the H-1 epiphyseal area and still less in the H-3 diaphyseal part of the bone. The range of collagen values was greatest in the H-2 region with one animal (# 117) having almost 30% of the total dry weight as collagen.

In order to extract collagen, native and gelatin, from bone which represented progressively greater degrees of maturation, and therefore exhibited greater insolubility, several solvents were chosen which had previously been shown capable of breaking the noncovalent forces which stabilize the polypeptide chains. Except for one solvent, 8 M urea at 37°, all of the extractions were made at 4° and in mild acid or at neutral pH according to the flow sheet, Figure 28 and Table 6, and using the apparatus shown in Figure 27.

The percent of organic matrix material recovered by extraction, dialysis and freeze-drying is given in Tables 7 and 8. Although there was approximately 20% collagen in dry bone, most of the protein was insoluble, suggesting this material to be highly crosslinked. Less than 1% collagen was dissolved by 0.5 M acetic acid (Tables 9 and 10), for all samples, indicating that all animals had approximately the same quantity of newly synthesized collagen in the bone.

The importance of pulverized bone as a starting material for collagen extraction should be emphasized here. Differences in the literature reports of the quantity of collagen extracted from similar tissues may be partially reconciled by the inhomogeneity of the bone sample (18; 33).

Table 6 All of the 34 bone samples analyzed were ground to 80 mesh size and the residues were sequentially extracted with the same solvents, under identical procedures and conditions as shown.

| Weeks | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Solvent | Acetic Acid | | Urea | Guanidine | Lithium Chloride | | | Potassium thiocyanate | | |
| Molarity | 0.5 | | 8.0 | 5.0 | 5.0 | | | 5.0 | | |
| pH | ca.3.5 | | 7.2 | 7.0 | 7.2 | | | 7.2 | | |
| C° | 4° | | 37° | 4° | 4° | | | 4° | | |
| Procedure | Rocker | | Rocker | | Shaker | | | Tubes | | |
| No.Extractions | 3 | | 3 | 3 | 3 | | | 3 | | |

Table 7

RECOVERY OF SOLUBLE ORGANIC MATRIX FROM FEMORAL BONE

| Bone Area Designation | Treatment Animal No. | Percent Material Solubilized by Extractants | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Acetic Acid | Urea | Guanidine Sup. | Ppt | LiCl | KSCN | Total Soluble | Insoluble Residue |
| Femur - $F_1$ | Exp. 117 | 0.98 | 6.36 | 1.81 | 0.66 | 1.20 | 1.25 | 12.26 | 21.20 |
| " | " 104 | 0.70 | 7.46 | 2.80 | 0.62 | 1.53 | 0.94 | 14.05 | 8.89 |
| " | " Ave. | 0.84 | 6.91 | 2.31 | 0.64 | 1.37 | 1.09 | 13.15 | |
| " | Con. 124 | 0.75 | 6.31 | 1.63 | 2.02 | 1.43 | 1.69 | 12.20 | 17.52 |
| Femur - $F_2$ | Exp. 117 | 0.70 | 5.08 | 1.74 | 0.25 | 0.97 | 0.65 | 9.39 | 17.29 |
| " | " 104 | 0.56 | 5.22 | 1.11 | 0.17 | 0.91 | 0.68 | 8.65 | 15.80 |
| " | " Ave. | 0.63 | 5.15 | 1.43 | 0.21 | 0.94 | 0.66 | 9.02 | 19.14 |
| " | Con. 124 | 0.92 | 6.03 | 4.62 | 1.54 | 1.29 | 1.28 | 15.69 | 15.54 |
| Femur - $F_3$ | Exp. 117 | 0.40 | 1.99 | 0.03 | 0.41 | 0.20 | 1.27 | 4.30 | 20.13 |
| " | " 104 | 0.61 | 2.14 | 0.05 | 0.42 | 0.31 | 1.03 | 4.56 | 22.14 |
| " | " Ave. | 0.51 | 2.07 | 0.04 | 0.42 | 0.26 | 1.15 | 4.45 | 21.14 |
| " | Con. 124 | 1.09 | 2.00 | 0.10 | 0.46 | 0.23 | 0.39 | 4.27 | 20.25 |

Table 8

RECOVERY OF SOLUBLE ORGANIC MATRIX FROM THE HUMERUS

| Bone Area Designation | Treatment Animal No. | Percent Material Solubilized | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Acetic Acid | Urea | Guanidine Sup. | Guanidine Ppt. | LiCl | KSCN | Total Soluble | Insoluble Residue |
| Humerus-$H_1$ | Exp. 106 | 0.28 | 3.92 | 0.30 | 2.45 | 0.43 | 2.40 | 9.78 | 22.80 |
| " | " 117 | 0.54 | 3.35 | 0.32 | 0.76 | 0.40 | 2.00 | 7.37 | 23.95 |
| " | " 104 | 0.68 | 5.14 | 0.32 | 1.47 | 0.24 | 2.28 | 10.13 | 18.42 |
| " | " Ave. | 0.50 | 4.14 | 0.31 | 1.56 | 0.35 | 2.22 | 9.08 | 21.72 |
| Humerus-$H_2$ | Exp. 106 | 0.79 | 3.78 | 0.14 | 0.89 | 0.39 | 1.43 | 7.42 | 22.56 |
| " | " 117 | 0.84 | 3.32 | 0.10 | 0.43 | 0.26 | 1.01 | 5.96 | 21.89 |
| " | " 104 | 0.98 | 4.26 | 0.16 | 0.70 | 0.36 | 2.01 | 8.47 | 20.11 |
| " | " Ave. | 0.87 | 3.78 | 0.13 | 0.67 | 0.33 | 1.48 | 7.28 | 21.85 |
| Humerus-$H_3$ | Exp. 106 | 0.64 | 2.14 | 0.05 | 0.56 | 0.26 | 1.19 | 4.84 | 18.64 |
| " | " 117 | 0.70 | 2.22 | 0.11 | 0.44 | 0.21 | 1.61 | 5.29 | 20.43 |
| " | " 104 | 0.78 | 2.66 | 0.09 | 0.34 | 0.23 | 0.93 | 5.03 | 19.67 |
| " | " Ave. | 0.71 | 2.34 | 0.08 | 0.45 | 0.23 | 1.24 | 5.05 | 19.51 |

Table 9

SOLUBLE ORGANIC MATRIX OF FEMORAL SWINE BONE

| SAMPLE | EXPERIMENTAL | | | | CONTROL | | | |
|---|---|---|---|---|---|---|---|---|
| FEMUR | mg/100mg Dry Bone | | | | mg/100mg Dry Bone | | | |
| | EXTRACTED | | CUMULATIVE | | EXTRACTED | | CUMULATIVE | |
| F-1 | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen | Dry wt.mg | % of Collagen | Dry wt.mg | % pf Collagen |
| Acetic | 0.84 | 3.95 | 0.84 | 3.95 | 0.75 | 3.52 | 0.75 | 3.52 |
| Urea | 6.91 | 32.45 | 7.75 | 36.38 | 6.37 | 29.20 | 7.12 | 33.42 |
| Guan: | | | | | | | | |
| Sup. | 2.31 | 10.85 | 10.06 | 47.69 | 1.63 | 7.65 | 8.75 | 41.07 |
| Ppt. | 0.64 | 3.00 | 10.70 | 50.23 | 2.02 | 9.48 | 10.77 | 50.56 |
| LiCl | 1.37 | 6.44 | 12.07 | 56.66 | 1.43 | 6.71 | 12.20 | 57.27 |
| KSCN | 1.09 | 5.12 | 13.15 | 61.78 | 1.69 | 8.09 | 13.89 | 65.36 |
| Tot. Sol. | 13.15 | 61.78 | | | 13.89 | 65.36 | | |
| Tot. Res. | 21.20 | | | | 17.52 | | | |
| Tot. Coll. | 21.30 | | | | 20.90 | | | |
| F-2 | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen |
| Acetic | 0.63 | 2.95 | 0.63 | 2.95 | 0.92 | 4.31 | 0.92 | 4.31 |
| Urea | 5.15 | 24.17 | 5.78 | 27.12 | 6.03 | 28.20 | 6.95 | 32.62 |
| Guan: | | | | | | | | |
| Sup. | 1.43 | 6.71 | 7.21 | 33.84 | 4.62 | 21.69 | 11.57 | 54.31 |
| Ppt. | 0.21 | 0.98 | 7.42 | 34.82 | 1.54 | 7.23 | 13.11 | 61.54 |
| LiCl | 0.94 | 4.41 | 8.36 | 39.23 | 1.29 | 6.05 | 14.40 | 67.60 |
| KSCN | 0.66 | 3.66 | 9.02 | 42.89 | 1.28 | 7.54 | 15.68 | 75.14 |
| Tot. Sol. | 9.02 | 42.89 | | | 15.69 | 75.14 | | |
| Tot. Res. | 16.55 | | | | 15.54 | | | |
| Tot. Coll. | 18.02 | | | | 16.98 | | | |
| F-3 | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen |
| Acetic | 0.51 | 2.35 | 0.51 | 2.39 | 1.09 | 5.11 | 1.09 | 5.11 |
| Urea | 2.07 | 9.71 | 2.58 | 12.11 | 2.00 | 9.30 | 3.09 | 14.50 |
| Guan: | | | | | | | | |
| Sup. | 0.04 | 0.18 | 2.62 | 12.30 | 0.10 | 0.46 | 3.19 | 14.97 |
| Ppt. | 0.42 | 1.97 | 3.04 | 14.27 | 0.46 | 2.15 | 3.65 | 17.13 |
| LiCl | 0.26 | 1.22 | 3.30 | 15.49 | 0.23 | 1.07 | 3.88 | 18.21 |
| KSCN | 1.15 | 6.03 | 4.45 | 21.52 | 0.39 | 2.09 | 4.27 | 20.26 |
| Tot. Sol. | 4.45 | 21.52 | | | 4.27 | 20.26 | | |
| Tot. Res. | 21.14 | | | | 20.25 | | | |
| Tot. Coll. | 19.08 | | | | 19.00 | | | |

Table 10

SOLUBLE ORGANIC MATRIX OF HUMORAL SWINE BONE

| SAMPLE | EXPERIMENTAL | | mg/100mg Dry Bone | |
|---|---|---|---|---|
| HUMERUS | EXTRACTED | | CUMULATIVE | |
| H-1 | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen |
| Acetic Acid | 0.50 | 2.34 | 0.50 | 2.34 |
| Urea | 4.14 | 19.43 | 4.64 | 21.78 |
| Guanidine: | | | | |
| Sup. | 0.31 | 1.45 | 4.95 | 23.23 |
| Ppt. | 1.56 | 7.32 | 6.51 | 30.56 |
| LiCl | 0.35 | 1.64 | 6.86 | 32.20 |
| KSCN | 2.22 | 10.40 | 9.08 | 42.60 |
| Total Soluble | 9.08 | | | |
| Total Residue | 21.72 | | | |
| Total Collagen | 21.34 | | | |
| H-2 | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen |
| Acetic Acid | 0.76 | 3.56 | 0.76 | 3.56 |
| Urea | 3.18 | 14.92 | 3.94 | 18.48 |
| Guanidine: | | | | |
| Sup. | 0.12 | 0.56 | 4.06 | 19.04 |
| Ppt. | 0.62 | 2.91 | 4.68 | 21.95 |
| LiCl | 0.33 | 1.36 | 5.01 | 23.31 |
| KSCN | 1.48 | 6.09 | 6.49 | 29.40 |
| Total Soluble | 6.49 | | | |
| Total Residue | 21.85 | | | |
| Total Collagen | 24.30 | | | |
| H-3 | Dry wt. mg | % of Collagen | Dry wt. mg | % of Collagen |
| Acetic Acid | 0.71 | 3.33 | 0.71 | 3.33 |
| Urea | 2.34 | 10.98 | 3.05 | 14.31 |
| Guanidine: | | | | |
| Sup. | 0.08 | 0.37 | 3.13 | 14.68 |
| Ppt. | 0.45 | 2.11 | 3.58 | 16.80 |
| LiCl | 0.23 | 1.16 | 3.81 | 17.95 |
| KSCN | 1.24 | 6.24 | 5.05 | 24.19 |
| Total Soluble | 5.05 | | | |
| Total Residue | 19.51 | | | |
| Total Collagen | 19.88 | | | |

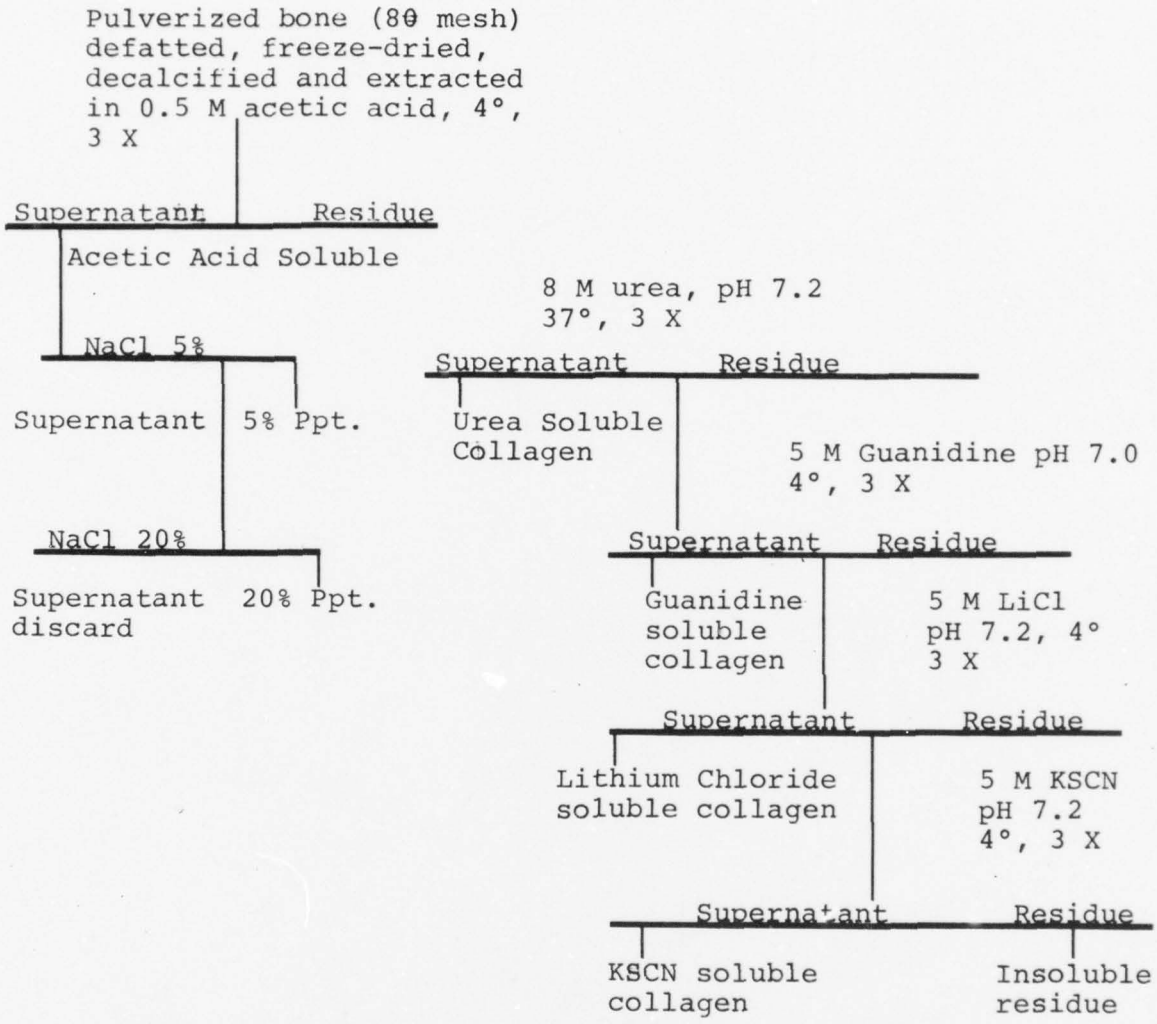
We found in preliminary studies that bone pieces and finely pulverized bone from the same tissue gave variable extractability of collagen, even after decalcification and homogenization of the pieces. Therefore, all of the results reported here were obtained from bone which had been finely ground after defatting.

The values (Table 9) for experimental and control are similar for most samples and extractants except for the F-2 region of the femur. In this area, guanidine extracted approximately 3 times more organic matrix from the control bone than from that of the experimental. However, in the humerus a striking sequential decrease was observed in going from the head to the shaft region with guanidine extraction.

The guanidine soluble fractions, although clear following extraction and centrifugation, formed a precipitate during dialysis. The resultant precipitate and supernatant fraction were separated by centrifugation prior to freeze-drying. The total guanidine extract was considered together for purposes illustrated in Figure 29. The guanidine supernatant and precipitate were treated separately for Tables 9 and 10. In the femur samples F-1 and F-2, more of the extracted organic matrix appeared in the supernatant fraction than in the precipitate following dialysis. However, in the femur F-3 and also samples of the humerus, 5 to 6 times greater weight was recorded in the precipitate.

As can be readily seen from Figure 29, recoveries of soluble matrix as the result of lithium chloride extraction are similar in the femur for all regions examined although

Figure 28 Flow Sheet for Collagen Extraction from Swine Bone Samples



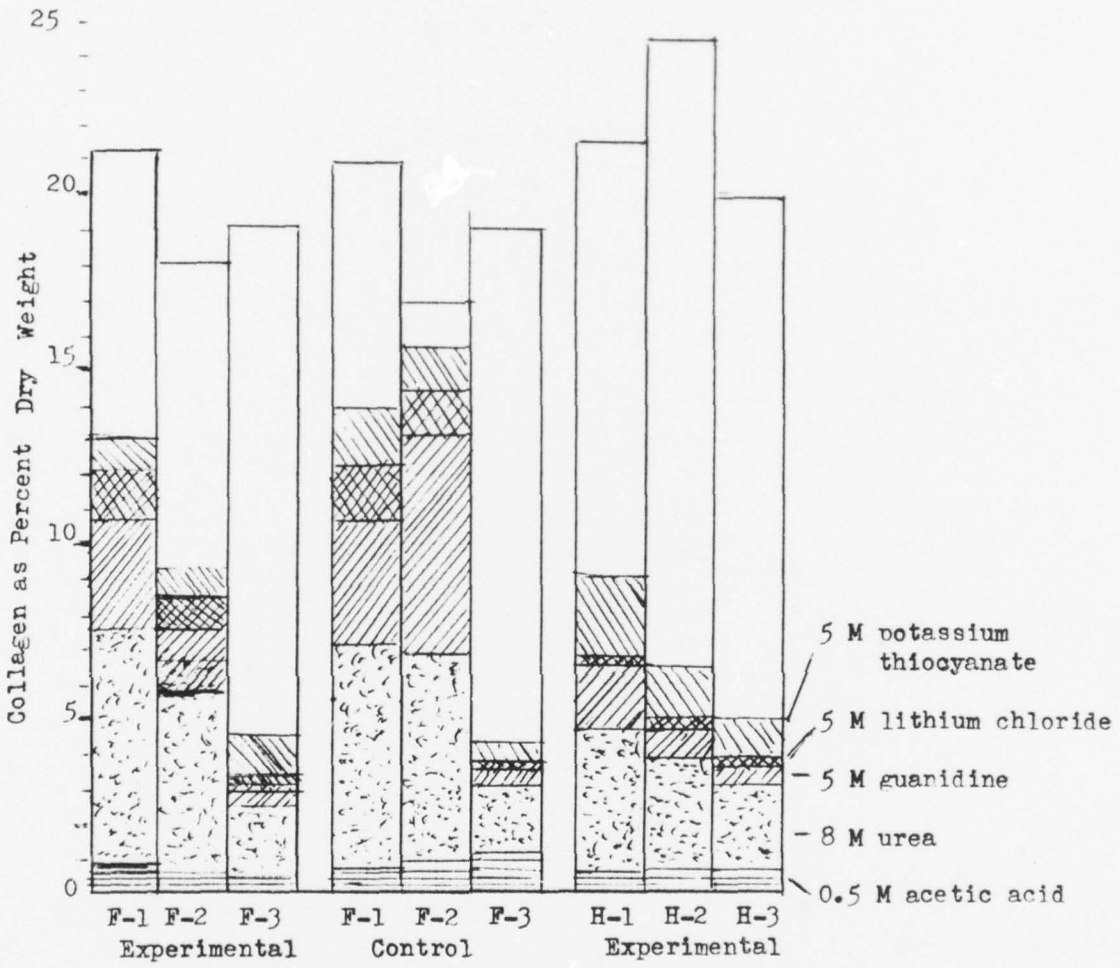



Figure 29 Percent recoveries of organic matrix of bone using successive extractions of (from the bottom) 0.5 M acetic acid, 8 M urea, 5 M guanidine, 5 M lithium chloride and 5 M potassium thiocyanate, with the conditions as described in the text. The upper bar represents total collagen contained in the samples. The femur and humerus sample designations are given in the text.

a decrease in extractable material was observed from the head toward the shaft region.

Recovery of organic matrix from whole bone using various denaturing or chaotropic agents is shown in Figure 28. The individual and the cumulative extractable quantities of organic matrix, which are principally collagen, are given in Tables 9 and 10.

The cumulative extractable collagen (Table 9) from the femur is greatest in the epiphyseal region F-1 for the experimental group with 62% of the total collagen solubilized. For the control bone, same region, the percent extractable is 65% but lower than the 75% extracted from the metaphyseal F-2 region. However, the experimental bone showed successively decreased solubility of collagen for the metaphyseal F-2 and the diaphyseal region F-3. Approximately 20% less collagen was solubilized in each area, 43 and 22% respectively. The control bone had the same cumulative extractable collagen, or 20%, in the diaphysis region as the experimental.

The insoluble residue remaining following the potassium thiocyanate extraction is given in Table 9. There does not seem to be pattern for this fraction as there was for the soluble organic matrix, although the range is about 15 to 20% for all bone areas analyzed. Tissue losses must be considered for both soluble and insoluble residue, for collagen is a very "sticky" substance (being the basis for many glues) and operational losses are inevitable. Even though the recovery of soluble organic matrix varied from

13, 9 and 4% for experimental bone F-1, -2 and -3, respectively, the recovered insoluble residue was found to be 21, 17 and 21%, respectively. Therefore the decreased solubility was not accounted for in the residue. We are continuing to analyze this material which should contain any elastin present from the vascular system.

Cumulative extractions of the humoral bones are given in Table 8 and Figure 29. The acetic acid soluble fraction is approximately the same for all bone areas. However, each succeeding extractant recovered successively less collagen as bone became distal to the epiphyseal region. The total soluble collagen decreased from 43 to 30 to 24% for H-1, -2 and -3 respectively.

Acrylamide gel electrophoresis has been run for some extracted collagen (Fig. 30). The monomer $\alpha$ chain is as indicated in the figure, with the more basic $\alpha_2$ chain migrating ahead of the $\alpha_1$ chains. Bone collagen contains 2 $\alpha_1$ per each $\alpha_2$ chain. The $\beta$ chains are not as well separated as the $\alpha$ but are visible approximately 1 cm behind. The trimer $\gamma$ component appears as 2 weakly staining bands about 1 cm into the gel.

The more difficultly soluble collagen fractions, such as guanidine, lithium chloride and potassium thiocyanate extractable, have stronger staining $\alpha$ than $\beta$ bands. This could be a difference in affinity for the dye, and other types of separation would have to be made to precisely quantitate the protein. However, it appeared that more $\alpha$ (monomer) chains

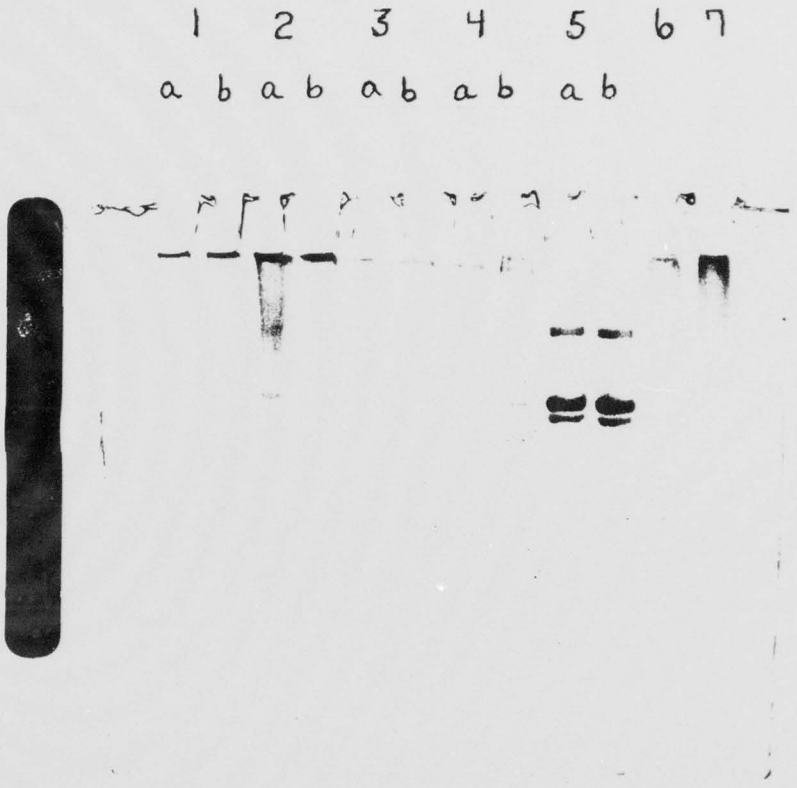



Figure 30 Slab electrophoresis, acrylamide gel 5%, 0.5 M urea, 0.1% SDS ( Goldberg et al. 1972 ) according to conditions given in text. Samples 1-4, 50µg each:
1. urea soluble, (a) experimental, (b) control;
2. guanidine supernatant, (a) experimental, (b) control;
3. lithium chloride, (a) experimental, (b) control;
4. potassium thiocyanate, (a) experimental, (b) control;
5. neutral salt soluble guinea pig skin for standard, (a) 25µg, (b) 50µg; 6. urea soluble, 1(b), 25µg;
7. guanidine supernatant, 2(b), 25µg.

than β (dimer) were extracted by denaturing solvents.

Gel electrophoresis patterns of the acetic acid extractable collagen indicated that the collagen had been degraded. This could possibly be attributed to the extensive dialysis (28 days) in 0.15 M acetic acid. This fraction was the only one having a protein staining band at the origin, indicating polymerization of the polypeptide chains during dialysis.

Total amino acid analysis of some of the extracted collagen from control bones has been determined (Table 11). The characteristic amino acid composition for collagen was seen, especially the high content of hydroxyproline. In general, hydroxyproline contributed about 10% of the total amino acid residues in the collagen molecule. Some differences in this figure occurred between species, tissue and genetic origin (20) of certain collagens. Another criterion for collagen is the high content of glycine, which contributes almost 33% of the amino acid residues. Presently, no other data is available for comparison of our results on femoral swine bone, and, although the glycine content appeared low when compared to that of collagen from growing rat bones, more experiments must be carried out to further characterize the swine bone collagen. All other values for collagen were in the approximate range expected for collagen with the exception of the hydroxy- and acidic-amino acid residues which appeared to be somewhat increased. It is of interest that these latter residues were those found in a high concentration in an acidic glycoprotein which is difficult

Table 11. Amino Acid Composition of Organic Matrix Soluble in Indicated Solution. Values are given as residues per 1000 residues. The conditions for hydrolysis are given in the text.

| | Acetic Acid | Urea | Guanidine Supernatant | Insoluble Residue | Insoluble Residue-Rat Bone* |
|---|---|---|---|---|---|
| Lysine | 39 | 30 | 31 | 30 | 34 |
| Histidine | 14 | 9 | 10 | 9 | 15 |
| Hydroxylysine | tr | 15 | 4 | 22 | 8 |
| Arginine | 46 | 56 | 51 | 53 | 53 |
| Hydroxyproline | 86 | 105 | 99 | 98 | 84 |
| Aspartic Acid | 69 | 62 | 68 | 62 | 53 |
| Threonine | 48 | 39 | 43 | 41 | 24 |
| Serine | 58 | 45 | 53 | 50 | 40 |
| Glutamic Acid | 90 | 87 | 94 | 87 | 76 |
| Proline | 105 | 121 | 116 | 115 | 102 |
| Glycine | 206 | 237 | 219 | 226 | 307 |
| Alanine | 112 | 93 | 107 | 94 | 94 |
| Half Cystine | -- | -- | -- | -- | -- |
| Valine | 35 | 26 | 30 | 27 | 29 |
| Methionine | tr | tr | tr | 3 | 7 |
| Isoleucine | 14 | 14 | 14 | 15 | 15 |
| Leucine | 43 | 35 | 35 | 37 | 34 |
| Tyrosine | 7 | 5 | 7 | 6 | 8 |
| Phenylalanine | 19 | 18 | 19 | 19 | 17 |
| Prehistidine | 4 | 5 | 4 | 5 | |
| Post-histidine | -- | tr | tr | -- | |
| Hexoseamine | 6 | tr | -- | | |
| Total Residues | 1001 | 1002 | 1004 | 1000 | 1000 |
| Proline/Hydroxy-proline | 1.22 | 1.15 | 1.17 | 1.18 | 1.22 |

* Deshmukh et al, 1973, values are for insoluble residue following extraction with 8 M urea, for growing rat bones.

to remove from connective tissue proteins from a wide range of tissues (61,45).

The aldehyde content of collagen is a little explored region of connective tissue metabolism but gradually is yielding to persistent and imaginative investigation, particularly of the bone collagen. Aldehydes are formed from either residues of lysine or hydrozylysine (52), and then proceed to crosslink to form an insoluble network. The extreme insolubility of bone collagen would suggest a high content of crosslinks. This is born out in work from other laboratories in which a high content of free aldehyde is found in 0.5 M acetic acid soluble bone collagen of growing rats (12). Approximately two-thirds less aldehyde was found in 8 M urea soluble extracts, however, and none in the insoluble residue.

C. Discussion

Two goals of this project have been to determine the relative efficacy of chaotropic agents as extractants for bone collagen and to develop methodology for such procedures. A third goal was to apply these procedures to bone samples from experimental and control animals to determine differences in collagen content and to identify possible changes in bone collagen metabolism. Differences in collagen content are defined operationally by extractability in solvents capable of breaking non-covalent bonds which stabilize the polypeptide chains of collagen.

This report describes the first systematic application, of which we are aware, for determining the collagen content and solubility of defined bone regions of swine femur and humerus from control and experimental animals with induced aseptic bone necrosis using solutions of increasing chemical activity.

Collagen in bone, as in most vertebrate collagen, is composed of 3 chains of approximately 100,000 MW each (34). As the single chain collegen molecules are synthesized and extruded from the cell, they undergo various changes and eventually are intertwined with 2 other chains to form a triple helix configuration which is probably unique to collagen.

Further chain interaction between the 3 polypeptides in the collagen molecule and polypeptide unites from other collagen molecules produces a crosslink system which becomes more and more difficult to dissolve. In bone, during this natural course of events, other compounds of the mucopolysaccharide class are synthesized as well and form, with collagen, the proper environment for calcification to occur. In newly formed bone, there is a larger quantity of organic matrix which is relatively soluble than in older bone. If, in some bone disorders such as hyperthyroidism or hyperparathyroidism, bone collagen is being broken down, then a lower content of collagen may be found, as well as increased excretion of the amino acid hydroxyproline (62;31). As a reaction to injury, certain cells are mobilized which can synthesize collagen and begin repair of the tissue. In this case, depending on the degree and severity of the injury, collagen may be synthesized in varying amounts. This collagen may be gradually resorbed or may be formed in such quantity as to result in fibrosis. Although increased collagen synthesis may occur as a reaction to an insult to tissue, the temporal aspect of the maintenance of a given collagen content must be considered before conclusions can be drawn regarding the significance of altered collagen content.

Involvement of epithelial collagen in initiating platelet aggregation is dependent on certain architectural requirements (28). Such platelet aggregation may be of importance in previously described histologic and hematologic changes (53)

in swine with induced aseptic bone necrosis.

Molecular oxygen is required for collagen synthesis (55; 40) for the hydroxylation of proline and lysine (52). Deprivation of oxygen for sufficient time periods may affect primary collagen synthesis. Bone collagen has a content of hydroxyproline similar to that of skin collagen or approximately 10% of the total amino acids. Hydroxylysine, however, is present in bone collagen from other species such as chick tibiae in higher quantities than in skin and in half the quantity of lysine or approximately 1.5% of the total amino acid residues. The hydroxyproline is important in giving the molecule the proper helical conformation. The high content of hydroxylysine in bone collagen appears to be unique and may be related to the high crosslinking capabilities as well as to the active remodeling which it undergoes (12).

Brown, et al. (5 ) found that total collagen in swine femoral proximal and shaft segments increased to a peak between 12 and 16 weeks but experiments were not carried past 24 weeks. At that time, most bone areas seemed to stabilize with respect to total collagen. These workers observed higher total collagen for the femoral proximal segment than for the shaft region. Chvapil, et al., (9 ) have determined that only in adult rats subjected to increased physical activity was there a significantly higher content of collagen found in the femur. We have found the same total collagen of around 20% dry weight in 3 year old swine for all areas of proximal femurs and humeri. However, in carrying our work

further by extraction of soluble collagen, dramatic differences have been obtained for the 3 bone areas analyzed.

Although the total quantity of bone collagen was similar, the solubility of collagen decreased in the diaphyseal region of both the femurs and humeri in contrast to the epiphyseal region. Rogers, et al., (48) found similar results with autoclave soluble collagen from rabbit bones. The exception in decreased solubility in swine was found in the metaphyseal region of the control femur in which increased solubility was noted for the guanidine soluble fraction. Solubility in guanidine is often associated with collagen having more stable crosslinks than the chains extractable by neutral salt or dilute acid solutions (7 ). The ratio of the dimer (β) chains to monomer (α) chains has been used to determine differences in the lability or stability of the collagen chain crosslinks (7 ). Quantitative comparison with the amount solubilized from control bones, together with the ratio of β/α chains, could be interpreted as an alteration in type of crosslinks for that particular kind of collagen. Final interpretation of such data would be dependent on further identification of specific amino acid residues involved in the chain crosslinks.

Acrylamide gel electrophoresis separated the $\alpha_1$, $\alpha_2$, and $\beta_{11}$, $\beta_{12}$ chains and some γ constituents. These analyses are only partially completed. Presently, the results indicate greatest percent extraction of α and β chains with 8 M urea. The protein staining band at the origin of the acetic acid

extracts indicates that further crosslinking occurred during the in vitro procedures for isolation of the collagen, and that the sub-units formed may be too high MW to migrate into the gel.

No such bands were seen at the origin for extractions of collagen with other solvents. The lithium chloride extraction produced weakly staining bands in the $\alpha$ and $\beta$ regions, and no other visible bands, although the same quantity of protein by weight was applied to the gel.

Further extraction of the organic matrix of the bone with potassium thiocyanate produced bands which stained in the $\alpha$ and $\beta$ chain region of the acrylamide gel slab but no bands were observed in the $\gamma$ chain region. The question as to whether collagen chains extracted by salts of high chemical activity such as guanidine, lithium chloride and potassium thiocyanate are degraded or not was substantially negated by Eyre and Glimcher (15). Recent evidence from their laboratory supports the contention that solubilization was mediated by disruption of relatively labile intramolecular and intermolecular bonds. The evidence identifies those bonds as labile aldimine crosslinkages, arising from aldehyde formed from lysine and hydroxylysine residues.

The low content of trimer and higher polymer chains indicated that substantially the same molecular weight and degree of crosslink was in the dimer and monomer form extracted by potassium thiocyanate or urea, as by acetic acid. The difference between the extracted material will be in the type of crosslink which held the collagen molecules stable

and prevented their extraction by the previous solvent. The aldehyde content of these fractions will be of great importance as differences in solubility must be accounted for by this means. That a substantial quantity of collagen is extracted with urea, guanidine, lithium chloride and potassium thiocyanate indicates that much of the remodeling of collagen is attributed to these fractions. It is also of interest that urea extractable collagen from both the femur and humerus decreased in the diaphyseal region to approximately one-third that of the epiphyseal region, for control and experimental alike.

The quantity of potassium thiocyanate extracted collagen remained similar for the experimental femur in all regions, but in the control femur decreased in the same proportion as urea soluble collagen for the diaphyseal region. An interpretation might be that in the experimental femoral diaphysis crosslinking of potassium thiocyanate extractable collagen was inhibited. In the experimental humerus an observation of the urea and potassium thiocyanate soluble collagen presented the same profile as the femur. Aldehyde analyses of these samples and quantitative densitometer tracings of the acrylamide gel electrophoreses will enable us to better interpret these findings. Presently we can say that the newly soluble, acetic acid extractable collagen is similar for all samples. The decrease in the urea and guanidine soluble collagen accounts for the major decrease in solubility for all bone samples.

Our preliminary findings of increased hydroxylysine content with progressively more insoluble collagen are the opposite of those reported by Deshmukh, et al., (12) for growing rat bone. Our findings would be consistent with the theory that hydroxylysine residues contribute to increased crosslinks, and therefore increased insolubility (12).

In contrast to what occurs with rat skin under normal oxygen (12), incubation of soluble bone collagen at 37° for one week resulted in no new crosslinks to form β collagen. After incubation of skin collagen at 150 psi at 37° for 4 weeks, similar decreased solubility was observed (43) in contrast to soluble bone collagen.

Many crosslinks are formed through aldehydes that are derived from lysine or hydroxylysine. These aldehydes can then condense with the ε-amino groups of lysine or hydroxylysine with other aldehydes to generate a possible six crosslinking structure that may link two protein chains. Some of these crosslinks can condense further to structures that may be capable of linking three or even four chains. The characterization of these crosslinks is often difficult because of their instability in acid or base. Reduction with radioactive labelled compounds such as tritium labelled sodium borohydride ( 4) or cyanohidridoborate (32), and carbon-14 labelled sodium cyanide in ammonia (39) can mark these reducible crosslinks.

The results obtained from our analyses of necrotic areas of femoral and humeral bone suggest approaches which should

be made in understanding changes which occur in this syndrome. The first approach is to identify covalent and reducible crosslinks of bone collagen using the procedures recently described by Mechanic (32), Pereyra, et al., (39) and Deshmukh, et al., (12). Briefly, the procedures measure the incorporation of radioactive label into reducible crosslinks of connective tissue proteins. Crosslinks which were formed in the collagen molecule and reduced to covalent bonds in vivo would not contain any radioactivity. Crosslinks which were stabilized by the procedure with radioactive reducing agents in vitro would be detected by scintillation counting and identified by amino acid analysis, thus determining the specific activity. This approach would provide information regarding the state of collagen extracted with denaturing solutions which would be valuable in defining the metabolic condition of bone collagen in aseptic bone necrosis. The $\alpha$, $\beta$ and $\gamma$ sub-unit quantities and their aldehyde content, which will be completed in the coming weeks, provides a substantial foundation for identification of crosslinks in bone collagen which leads to further work regarding the reduction procedure.

Another approach would be to obtain direct information of the biosynthetic state of bone collagen synthesis in vitro. This procedure would be similar to that presently used for chick embryo cranial bone (35;58) and would determine rates of radioactive proline uptake and conversion to hydroxyproline.

Identification of crosslinks which are covalent *in vivo* and those which may be reduced to stable covalent crosslinks *in vitro* will give information which may account for extreme insolubility of bone collagen. Metabolic differences in the tissue brought about by the present diving procedures may be identified with a more thorough understanding of normal maturation of collagen, coupled with knowledge of bone cells' capability to repair collagen which has been destroyed or modified.

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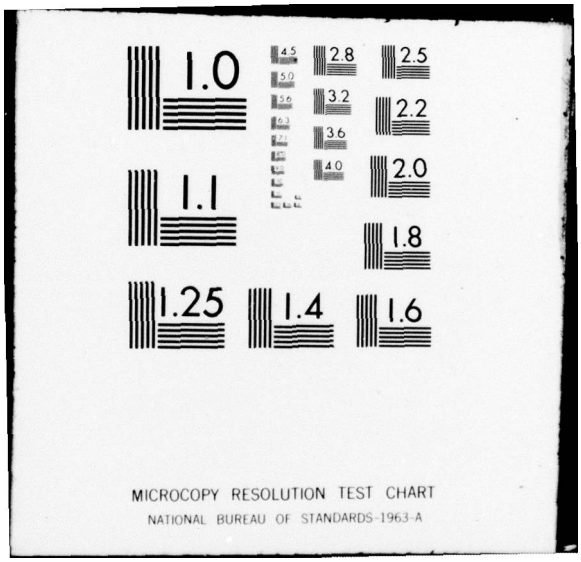
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MICROCOPY RESOLUTION TEST CHART
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2. Smith, K.H. Aseptic Bone Necrosis Production and Evaluation in Miniature Swine. (Presented at Working Conference on Radiological Aspects of Aseptic Bone Necrosis Related to Diving, Bethesda, Md., Feb., 1972).

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Knowledge of the pathology associated with inadequate decompression is truly embryonic. In that the Bureau of Medicine and Surgery, U.S.N., and Office of Naval Research saw fit to support our investigative effort, significant information was gathered which describes certain pathological mechanisms and hints at the description of etiological processes.

Our investigations evaluated the response of 67 miniature swine to

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4760 hours of simulated diving in our chambers. The study included the testing of 1404 blood samples for kinetic measurements of hemostatic mechanisms, 425 radiographs for detection of bone disease and numerous histologic slides for microscopic examination.

Some of the information generated revealed the importance of endothelial integrity, its susceptibility to injury following inadequate decompression and its effect on the hemostatic mechanism and surrounding tissues when they are damaged. Two simultaneous but separate studies demonstrated the importance of collagen in the pathogenic process of osteonecrosis.

Of significance is that this pathologic process was not limited to bone tissue, but probably occurred throughout the body. Only the manifestation seems to differ in both degree and character.

Certain evidence accumulated appeared important and unequivocal:

1) Dysbaric osteonecrosis was produced in miniature swine following a single decompression exposure.
2) Hemostatic changes seen in diving animals and humans were the result of the decompression process, and more specifically, the relative tissue inert gas tension.
3) The manifestation of the hemostatic changes were blocked by the administration of antithrombotic medication in both animals and human divers.
4) Blocking of the hemostatic mechanism changes with antithrombotic medication did not prevent the occurrence of osteonecrosis.
5) Tissue injury occurred with the first decompression insult and was aggravated by repeated exposure.
6) Severe endothelial and small valcular damage occurred following compression/decompression exposures which did not produce signs of decompression sickness.

Other data we accumulated suggested certain pathological events or relationships but insufficient information was available for confirmation. They suggest that:
1) Bubbles passing through a vessel interact with the endothelium to produce the changes that were seen.
2) Antithrombic medication facilitates the production of decompression-related osteonecrosis.
3) Exposure of basement membranes collagen is instrumental in producing hemostatic changes.
4) Collagen crosslinking is different in animals exposed to inadequate decompression than in normal controls.